FORAGE QUALITY AND BLOOD METABOLITES OF HORSES GRAZING ALFALFA, COOL-SEASON PERENNIAL GRASS, AND TEFF

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Michelle Leigh DeBoer

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Marcia Hathaway
Krishona Martinson

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FORAGE AND HORSES

Meeting the nutritional requirements of the horse is not only crucial for the health and wellbeing of the animal but can also help maximize growth and performance. Nutrients can be classified into six primary categories: carbohydrates, protein, fat, vitamins, minerals, and water. While feed companies will often balance these rations in the form of grain or concentrates, forage is the primary food source in the horse's diet. The importance of forage can largely be contributed to the physiological and anatomical traits of the digestive system of the horse, specifically relating to the size and acid secretion in the stomach. Horses have a rather small stomach, consisting of only 10% of the digestive tract volume. As a result, passage rate through the stomach is relatively limited with digesta remaining in the stomach for only two to six hours (Weyenberg et al., 2006). The small size and fast passage rate can pose a problem as the horse's stomach continuously secretes hydrochloric acid (HCl). Fortunately, the presence of ingesta and saliva, produced through chewing, act as a buffer for the low pH to combat this problem (Campbell-Thompson and Merritt, 1987). As a result, consuming small amounts of forage continuously throughout the day is important to maintain health and reduce the risk of colic (Murray, 1992), gastric ulcers (Andrews and Nadeau, 1999; Murray and Schusser, 1993), and wood-chewing behavior (Willard et al., 1977) in horses.

Forage is often consumed by horses in two forms: pasture or hay. The key differences between these forage types includes the stage of maturity and dry matter

(DM) content. In regards to maturity, hay is often utilized at a more mature stage when compared to pasture in order to increase harvesting yields (Weir et al., 1960). However, increased maturity is also associated with decreased nutritive values, specifically, increased fiber (Harkin, 1973), which reduces digestibility of the feedstuff, and decreased crude protein (CP; Palmonari et al., 2014).

The other key difference we observe between hay and fresh pasture is the DM content. Considering hay is a dried and preserved form of pasture, hay has a higher DM content in comparison to pasture. Unfortunately, as a result of the drying and preservation techniques, hay may have decreased nutritive quality resulting from respiration, rain damage, mechanical damage, and storage losses. Respiration in plants, a process producing carbohydrates to be utilized for energy and growth, continues after harvest until the plant is dried or stores have been depleted. The continuation of this process can result in significant losses of soluble carbohydrates to decrease the nutritive value of the hay (Rotz and Muck, 1994). Nutrient losses can also result from rain damage, as water-soluble nutrients, including CP, nonstructural carbohydrates (NSC), and water-soluble vitamins, will leach out of the forage (Fonnesbeck et al., 1986). Further nutrient losses may be observed by mechanical damage. These losses are likely a result of increased leaf loss or shatter as the leaves contain higher concentrations of most nutrients (Mowat et al., 1965; Rotz and Muck, 1994). Nutrient loss will also occur during storage specifically in nutrients including vitamin A and E which can become depleted (Bruhn and Oliver, 1978).

Despite the concerns of decreased nutritive quality, hay has the ability to provide forage to horses during the times of year when forage cannot be grown and utilized in

pasture systems. However, over 80% of horses have some access to pasture (NAHMS, 1998) with 87% of horses having access to pasture in Minnesota (Martinson et al., 2006). Considering fresh pasture is approximately one-third the cost of purchasing hay (McCormick et al., 2006), a properly managed pasture can be utilized as cost-effective and nutritious source of forage (NRC, 2007).

Forage Species

Regardless of whether forage is fed in the form of hay or pasture, there are many differences across forages which vary by different classifications. These classifications can include perennials and annuals, legumes or grasses, as well as photosynthetic pathways such as C_3, and C_4.

Annuals and Perennials. Classification as an annual or perennial is determined by the life of the plant. Perennial forages live more than two years in comparison to the shorter-lived annuals, which only last a single growing season. Cool-season perennial grasses are the foundation of horse pastures in the upper Midwest. This is because, in general, the greatest benefits are offered by perennials as they can be high yielding under proper management and do not need to be reseeded annually.

Research by Allen et al. (2012) evaluated cool-season perennials under horse grazing in Minnesota, including tall fescue, meadow fescue, quackgrass, smooth bromegrass, meadow bromegrass, reed canarygrass, perennial ryegrass, timothy, Kentucky bluegrass, creeping foxtail, and orchardgrass. This study observed yields ranging from 5.0 to 13.9 Mg ha^{-1} over the two-year study with the highest yields consistently observed in orchardgrass. Further research by Martinson et al. (2016) observed yields in cool-season perennial mixtures ranging from 6.1 to 7.1 Mg ha^{-1}. Allen

et al. (2012) also evaluated horse preference in cool-season grass species, measured by percent removal, and observed the greatest preference for Kentucky bluegrass, timothy, and meadow fescue.

Despite the benefits offered by perennials, annual grasses have demonstrated the ability to be effective as pasture supplementation when perennial pastures have decreased production. In research performed by Grev et al. (2017), annual cool-season grasses, specifically annual ryegrass, had adequate nutritive values, were highly preferred, and had yields ranging from 2.7 to 5.9 Mg ha^{-1}. Additional research evaluated warm-season annual grasses in horse pastures in the upper Midwest with yields ranging from 4.3 to 7.5 Mg ha^{-1} in teff as well as yields between 5.4 and 21.2 Mg ha^{-1} in sudangrass, the two most highly preferred annual warm-season grass species in this study (DeBoer et al., 2017). These studies demonstrate the ability of annual forages to be productive and palatable to horses. They also demonstrate the potential of annual forages to be a grazing alternative for horses in order to allow perennial pastures to rest during the summer slump (Riesterer et al., 2000), increase overall productivity, extend the grazing season, or offer emergency forage options when they are required.

Grasses and Legumes. Annuals and perennials can further be classified into grasses or legumes, which are identified based on anatomical structures and physiological qualities of the forage. Grasses, such as timothy, orchardgrass, Kentucky bluegrass, and bermudagrass are monocotyledonous with jointed and sheathing leaves and produce grain in the reproductive stage. In comparison, legumes, such as alfalfa and clover, are known for their ability to form symbiotic relationships with nitrogen-fixing bacteria called rhizobium. Other distinguishing characteristics include being dicotyledons, having

decreased structural matter in their leaves, increasing the stem to leaf ratio with maturity, and producing pods when they reach a reproductive stage.

One of the most striking benefits in legumes in comparison to grasses is their increased nutritive quality, specifically observed by increased digestible energy (DE), crude protein (CP), and calcium (Ca) content. In research evaluating various alfalfa varieties grazed by horses, DE was approximately 2.6 Mcal kg^{-1} (Catalano et al., 2015) in comparison to cool-season annual grasses with DE values around 2.3 Mcal kg^{-1} (Grev et al., 2017) and warm-season annual with DE values at approximately 2.1 Mcal kg^{-1} (DeBoer et al., 2017). When evaluating CP in alfalfa grazed by horses, values ranged from 28.5 to 29.5% DM (Catalano et al., 2015) in comparison to cool-season perennial grasses which had CP values ranging from 15.9 to 23.6% (Allen et al., 2013). Further research compared nutrient composition of alfalfa, tall fescue, and Caucasian bluestem hay fed to Arabians and found CP at 19%, 11%, and 7%, respectively (Crozier et al., 2011). This study also found Ca to be at 0.94% in alfalfa in comparison to 0.26% and 0.27% for tall fescue and Caucasian bluestem, respectively. Research performed in fresh pasture evaluated, the Ca to phosphorus (P) ratio, which was <1:1 in cool-season annuals (Grev et al., 2017) and > 3.2:1 in alfalfa (Catalano et al., 2015). As a result, legumes may be beneficial for horses with higher energy and nutrient demands such as performance horses and pregnant or lactating mares (NRC, 2007).

Photosynthetic Pathways. Forages can further be broken down by their photosynthetic pathways, which are most commonly classified as either C_3 or C_4. The key difference between these forages is their ability to handle different climates with C_3 species surviving better in temperate climates while C_4 forages prosper in sub-tropical or

tropical areas. The varying growth rates at different temperatures can be attributed to their unique photosynthetic pathways with cool-season grasses classified as C_3 and warm-season grasses classified as C_4.

Virtually all trees, shrubs, herbs, and cool-season grasses utilize the most basic and primitive photosynthetic system referred to as the C_3 pathway, or more commonly known as the Calvin cycle. This system initially converts carbon dioxide and ribulose biphosphate into a 3-carbon acid (Cerling, 1993). The most efficient carbon fixation when using the C_3 pathway occurs between 16 and 24°C (Fry and Huang, 2004). This is because RuBisCO (ribulose-1,5-biphosphate carboxylase/oxygenase), an enzyme involved in the first major step of carbon fixation, incorporates more oxygen into RuBP (ribulose-1,5-biphosphate) as the temperature rises above 24°C and causes CO_2 levels inside the plants to become low. As a result, photorespiration occurs and the stomata are forced to close to prevent water loss and the grasses go dormant as a result of a net loss of carbon and nitrogen.

In comparison, the C_4 pathway, commonly utilized in warm season grasses and sedges, experiences the most efficient carbon fixation between 30 and 35°C. This pathway combines CO_2 with phosphoenol pyruvate to form the 4-carbon acids malate and aspartic acid (Cerling, 1993). The grasses that utilize this pathway have a special anatomy with an abundance of bundle sheath cells, which surround the leaf vascular bundles (Fry and Huang, 2004). While there are similarities between the initial carbon fixation of the C_3 and C_4 pathways, C_4 plants have evolved to create high concentrations of CO_2 inside the bundle sheath cells to interact with RuBisCO in the Calvin-Benson cycle as the temperatures rise (Fry and Huang, 2004; Sage, 2004). This allows

photorespiration to be minimal at higher temperatures in comparison to cool-season grasses.

The climate in the upper Midwest is more suited to cool-season grasses, making them the predominant forage found in horse pastures. In comparison, perennial warm-season grasses are unable to survive the winter in the upper Midwest. However, recent research evaluating annual warm-season grasses has shown potential for these forages to be utilized within an equine grazing system during the grazing season in Minnesota. In research performed by DeBoer et al. (2017), teff and sudangrass were high yielding, highly preferred, and demonstrated sufficient nutritive qualities for grazing horses. However, as a result of the increased presence of bundle sheath cells in C_4 grasses, fiber content is often greater in warm- versus cool-season forages (Moore and Mott, 1973).

Species Differences in Forage Nutritive Quality

The three main classes of forages grazed by horses include cool- and warm-season grasses as well as legumes. In addition to their anatomical and physiological differences, these forages also have distinct nutritive qualities. As a result, it is important to select the appropriate forage to meet the nutritional requirements of the grazing horse. Three nutrients that are commonly evaluated include fiber, NSC, and crude protein (CP) with protein further broken down into nonprotein nitrogen (NPN) and the amino acid (AA) profile.

Fiber. Cell wall contents of the plant, including hemicellulose, cellulose, and lignin, comprise the fiber concentrations of a plant. This component is often measured in two forms: neutral detergent fiber (NDF) and acid detergent fiber (ADF). Neutral detergent fiber is important as it measures the cell wall components of the forage

including hemicellulose, cellulose, and lignin. Acid detergent fiber, on the other hand, measures the least digestible portions of the cell wall which is limited to cellulose and lignin. These values are important because ADF and NDF are inversely related to digestibility and intake, respectively (Buxton, 1996; Reid et al., 1988).

Fiber components may vary based on the photosynthetic pathways utilized by the forage specie with warm-season grasses having higher fiber concentrations than cool-season grasses. These differences can be attributed to the different anatomical structures required by the unique photosynthetic pathways to operate within different temperature ranges (Moore and Mott, 1973). Specifically, warm-season grasses have a higher presence of bundle sheath cells. The thick-walled bundle sheath cells are the site of photosynthesis in warm-season grasses to minimize photorespiration in warm temperatures. While this is beneficial, the bundle sheath cells increase the fiber concentrations of warm-season forages which can impact the quality of the grass.

When evaluating legumes, a lower cell wall content is observed in comparison to grasses, leading to lower fiber concentrations in legumes. Anatomically, grass leaf blades form a lignified midrib to provide mechanical support, which is believed to result in the higher fiber concentrations. Additionally, increased cell wall components in the grass leaf blades make them less digestible than the leaves of legumes. As a result, we see higher fiber values in cool-season grasses when compared to legumes which can decrease forage quality (Moore and Jung, 2001).

These fiber patterns have been consistently observed in scientific studies evaluating different forages. In a study evaluating cool- and warm-season grass, as well as alfalfa hay, the highest ADF and NDF values were observed in the warm-season grass

followed by cool-season grass with alfalfa having the lowest fiber concentrations (LaCasha et al., 1999). Corresponding to these fiber values, organic matter digestibility was highest in yearling horses consuming the alfalfa hay, at 74%, in comparison to those consuming the cool- or warm-season grass hay at 64% and 60%, respectively.

When evaluating pasture forage, lesser amounts of both ADF and NDF were consistently observed in cool- compared to warm-season grasses as well (Moore et al., 2004; Reid et al., 1988). One specific study (DeBoer et al., 2017) evaluated warm-season annual grasses compared to a cool-season annual grass control (annual ryegrass) under horse grazing in the upper Midwest. This research reported NDF values in vegetative forage > 57% in warm-season annual grasses and < 54% in annual ryegrass. In a separate study evaluating cool-season grasses compared to alfalfa, lower NDF values were observed in alfalfa and clover when compared to grasses such as orchardgrass and smooth bromegrass (Sleugh et al., 2000).

As mentioned previously, these fiber values are important as they correlate to the digestibility and intake of the horse. While digestibility has not been evaluated in horses grazing cool-season grasses, warm-season grasses, or legumes, research performed in sheep and cattle observed a decrease in dry matter digestibility (DMD) for animals consuming warm- compared to cool-season grasses (Reid et al., 1988). In this study sheep had a DMD of 54.6% and 61.7% while cattle had a DMD of 59.8% and 66.9% when grazing warm- and cool-season grasses, respectively. Additionally, in this study warm-season forages had a higher ADF value at approximately 43% while cool-season grasses had ADF concentrations around 37%. However, due to differences in the digestive systems of ruminants, such as sheep and cattle, in comparison to monogastric

hindgut fermenters, such as the horse, it will be important to evaluate the relationship between ADF and digestibility in grazing horses.

In addition to digestibility, increasing NDF has commonly been associated with a decrease in intake. As a result, a decreased voluntary intake would be expected in horses consuming warm-season grasses in comparison to cool-season grasses as decreased NDF values are commonly observed in cool-season grasses (Reid et al., 1988). While these differences in intake have yet to be evaluated in grazing horses, horses consuming the high fiber teff hay observed decreased intake in comparison to horses consuming cool-season grass hay (Askins et al., 2017, McCowan et al., 2012). These results support the relationship between intake and fiber concentrations.

While intake and digestibility are important considerations, they also have implications in metabolic concerns. The importance of digestibility is, in part, contributed to its relevance to the glycemic and insulinemic response in horses. This is observed as studies have determined decreased digestibility may lead to a decreased glycemic and insulinemic response in horses (Richards and Kempton, 2016). Additionally, intake is an important factor to consider as decreased intake has also been associated with an attenuated glycemic and insulinemic response in horses (Rodiek and Stull, 2007). A decrease in voluntary intake also has potential to decrease energy intake and ultimately lead to weight loss which would be advantageous in treating obese horses.

Nonstructural Carbohydrates. Nonstructural carbohydrates are an important nutritive value and are calculated by using the sum of water soluble carbohydrates (WSC) and starch. The overall composition of this nutritive component is fructans, simple sugars, and starch and can relate to the glucose and insulin response of a feed. While

NSC concentrations are often highest in grains and concentrates, forages can include a wide range of NSC concentrations as a result of diurnal variation, seasonal changes, and forage species.

In plants, NSCs are the main product of photosynthesis and provide substrates to be utilized for metabolism and growth. However, when NSCs are synthesized in excess, they can be converted into storage carbohydrates; which are fructans for CSGs and starch for WSGs and legumes. The NSC value of a plant will be determined by the difference between synthesis, via photosynthesis, and utilization, via growth and metabolism. An imbalance between synthesis and utilization can lead to NSC accumulation where photosynthesis outpaces the rate at which NSCs are utilized. As a result, diurnal variation can play a strong role in the NSC concentrations of a forage. This occurs as NSC values begin to rise in the morning, when photosynthesis begins, and peak in the afternoon. However, when the sun goes down and photosynthesis ceases, NSCs accumulated during the day can be utilized with NSC values decreasing overnight.

This relationship between NSCs and diurnal variation has been observed in numerous research studies. In a study (Lechtenberg et al., 1971) evaluating NSCs in alfalfa, glucose and fructose percentages rose from 06:00 to 12:00 and sucrose values rose between 06:00 and 18:00. Overall, all NSCs began to accumulate after 06:00. Similarly, research evaluating the diurnal variation of grasses (Holt and Hilst, 1969), including Kentucky bluegrass, bromegrass, and tall fescue, observed linear increases in water-soluble carbohydrates (WSC) percentages from 06:00 to 18:00. However, more rapid increases were observed in bromegrass and tall fescue in comparison to Kentucky bluegrass.

Further variation in NSCs can be observed across seasons due to differences in seasonal demands of the forage. Similar to diurnal variation, when photosynthesis outpaces utilization, NSCs can accumulate in the forage. As a result, highest NSC concentrations are consistently observed in the spring, intermediate values observed in the fall, and the lowest values observed in the summer (Longland and Byrd, 2006). These trends are the result of high light intensity observed concurrently with low temperatures in the spring and fall. This combination is a problem as high light intensity increases the accumulation of NSCs; however, enzymatic activity required for plant growth is compromised when temperatures fall below 4°C.

In regards to NSC in different forage species, substantial differences have been observed in cool-season grasses compared to warm-season grasses and legumes. While minimal research has reported the NSC values in warm-season grass and legume pastures, these classes of forage are known to have a lower NSC content; this trait can likely be attributed to their unique carbohydrate storage mechanism. Cool-season grasses store excess carbohydrates in the form of fructans which can be translocated to the stem. In comparison, warm-season forages have a self-limiting carbohydrate storage mechanism where carbohydrates are stored as starch in the chloroplasts (Longland and Byrd, 2006), contributing to the lower concentrations of NSC.

Research has confirmed these differences as a study evaluating teff hay consumed by horses found NSC contents ranging from 5% to 8% DM (Staniar et al., 2010) while DeBoer et al. (2017) found teff pastures were <9% DM. In comparison, cool-season perennials have reported values ranging from 6 to 17% (Allen et al., 2013) while cool-season annual grasses had NSC values ranging from 10 to 22% DM (Grev et al., 2017).

Despite the higher digestible energy (DE) values in legumes, alfalfa has NSC levels comparable to warm-season grasses due to the same carbohydrate storage mechanisms. In research evaluating grazing horses on alfalfa and clover, NSC concentrations ranged from 8 to 11% DM (Catalano et al., 2015). Similarly, Rodiek and Jones (2012) determined that alfalfa and teff hay had NSC ≤12% while oat and wheat hay had NSC values ≥30% DM. Considering a low NSC diet is considered less than 10 to 12% NSC (Borgia et al., 2009; Frank et al., 2009), teff hay falls into the acceptable range to be considered a low NSC forage.

Crude Protein. Protein is another important nutrient for the horse and is often measured in forages in terms of CP, which is a value based on the nitrogen content of the feed. However, nitrogen can be found in feeds in various other forms such as free amino acids (AA), nitrates, amides, and amines, all of which are classified as non-protein nitrogen (NPN) but still included within the CP measurement. As a result, measuring the protein quantity and quality is more accurately measured in terms of AAs for hindgut fermenters such as the horse. However, considering this value is rarely available, it is important to understand the CP and NPN differences across forages, as well as the AA content when presented.

The CP content, as well as the overall presence of nitrogen, can be influenced by many factors including growing conditions, level of nitrogen fertilization, maturity (Salon et al., 2001), and time of year. Overall, nitrogen use by plants requires several steps including uptake, assimilation, and translocation with more efficient processes observed in legumes. Improved efficiency in legumes compared to grasses is observed during the nitrogen fixation process. While grasses are dependent on nitrogen from nitrogenous

fertilizer sources, legumes can also convert atmospheric nitrogen to a form which can be used by the plant (Mus et al., 2016). As a result, CP is often higher in legumes, such as alfalfa, in comparison to grasses.

However, further differences can be observed when evaluating grasses as higher CP concentrations are commonly found in cool- compared to warm-season grasses. These results are likely observed due to the inverse relationship between CP and fiber. As fiber increases, in times such as increased maturity or photosynthetic pathway, decreases in CP are observed. This relationship suggests fiber competes with protein for either space or resources within the plant.

These differences across forage species have been observed in numerous studies including a study performed by Balde et al. (1993). This research project evaluated alfalfa and orchardgrass hay fed at one of four maturities to cattle. This study found that increased maturity resulted in increased fiber and decreased CP. However, despite these changes in nutrient profiles, the relative proportions of AAs were not altered.

A separate study (Elizalde et al., 1999) evaluated the composition of fiber and CP of alfalfa, bromegrass, and endophyte-free and –infected tall fescue forages at maturities ranging from vegetative to reproductive stages. The results from this study determined forage species had a strong impact on protein and fiber concentrations while maturity impacted certain CP and fiber fractions, specifically NDF, in the plants. Additionally, CP concentrations varied across species with higher values commonly observed in legumes compared to grasses.

These differences in CP across forage species have been observed in other studies. When Gibbs et al. (1988) evaluated different hay species fed to horses including

coastal bermudagrass (warm-season) as well as low- and high-protein alfalfa. These results demonstrated higher protein values in low- and high-protein alfalfa at 15.0 and 18.1% DM, respectively in comparison to coastal bermudagrass at 11.7% DM.

Another study performed by Woodward et al. (2011) evaluated timothy and alfalfa hay harvested at different maturities. These results indicated timothy had lower CP at 7.3% DM when compared to all maturities of alfalfa which exceeded 18.4% DM. Additionally, decreased CP was observed as alfalfa increased maturity, falling from 24.4% DM at early bud to 18.4% DM at mid-bloom.

Further research compared alfalfa, tall fescue and Caucasian bluestem hay fed to horses (Crozier et al., 1997). This study confirms the previous results with highest CP found in alfalfa at 19% DM, and followed by tall fescue and Caucasian bluestem at 11 and 7% DM, respectively. Additionally, LaCasha et al. (1999) evaluated alfalfa, Matua bromegrass, and coastal bermudagrass; alfalfa had the greatest CP at 20% in comparison to 13.5% and 11.3% in bromegrass and bermudagrass, respectively.

Finally, Staniar et al. (2010) observed a similar trend in teff hay regarding CP alterations with maturity. This study recorded CP values at 16.4, 10.8, and 7.5% DM for boot, early heading, and late heading stages, respectively.

However, these results are not consistent across all research with greater values observed in teff hay when compared to timothy at 14 and 11%, respectively (McCowan et al., 2012). However, CP can be influenced by other factors such as nitrogen fertilization and harvest dates. Previous research (Johnson et al., 2001) has observed increased protein fractions as a result of increased nitrogen fertilization as well as an

alteration of protein fractions as a result of harvest dates, factors that will be discussed further in relation to NPN.

Nonprotein Nitrogen. As mentioned previously, CP is an extrapolation of nitrogen and contains both true protein as well as NPN. This value poses a problem for monogastrics, such as horses and pigs, as they cannot utilize NPN, unlike ruminants. This is possible for ruminants as NPN can be utilized to synthesize essential AAs via fermentation in the rumen which can be absorbed into the blood as the bolus travels through the GI tract. However, in horses, protein and AAs are digested and absorbed in the foregut; which is limited to the stomach and small intestine. Any AAs that escape digestion and absorption in the foregut will be converted into microbial protein in the hindgut but are not absorbed and utilized in physiologically relevant quantities. So while the NPN constituent of CP is useful for ruminants, it is not applicable to protein requirements in horses. As a result, it is valuable to understand how this protein fraction changes as a result of various factors in order to account for it appropriately.

Some factors that can influence NPN include fertilization, harvest dates, and forage species. In research performed by Elizalde et al. (1999), NPN did not differ as a result of forage species or maturity in fresh forages including alfalfa, bromegrass, and tall-fescue. However, NPN averaged 22.6% of CP, demonstrating that approximately one quarter of CP does not contribute to protein requirements in monogastrics. Despite the minimal differences observed in NPN in this study, true protein was higher in alfalfa in comparison to the grasses.

Further research (Palmonari et al., 2001) evaluated the impact of harvest-intervals on protein fractions in alfalfa. This study found that shorter harvest-intervals had

increased protein, specifically via increased CP, soluble protein, and NPN. These results are evident as NPN concentrations for 21-d harvest intervals were 5.3% DM compared to 28 and 35-d intervals in which values dropped down to 3.5 and 3.9% DM, respectively. Despite higher overall CP levels observed during 21-d harvest intervals, NPN contributed to a higher percent of CP at 25% in comparison to 20 and 22% at 28- and 35-d, respectively.

Further research (Johnson et al., 2001) has evaluated the impact of harvest date and nitrogen fertilization on NPN concentrations in tropical grasses, including bermudagrass, bahiagrass, and stargrass. In this study, NPN, also referred to as fraction A, responded quadratically to harvest date with lower values observed in July and August when compared to early June. Additionally, a linear relationship was observed between NPN and nitrogen fertilization, with increased fertilizer leading to an increase in NPN. These changes were observed as NPN following fertilization with 157 kg N ha^{-1} rose from 31% to 40% of nitrogen while bahiagrass rose from 21.2% to 28.5% of nitrogen and stargrass rose from 27.9% to 42.1% of nitrogen. These results suggest that while increases in CP may be observed following nitrogen fertilization, these increases can largely be attributed to accumulation of NPN.

In addition to these factors that influence NPN, nitrogen-containing compounds (NCCs) can be influenced in plants as a result of stress; including but not limited to environmental stress and nutrient deficiencies. The NCCs include protein AA, nonprotein AA, amides, diamines, and polyamines. The type of NCC which will accumulate is dependent on the plant type and species as well as the nature of the stress. Specific examples include stress-induced proline accumulation as a result of water deprivation or

extreme salinity (Hare and Cress, 1997). Cadmium contamination in the soil can lead to increased free AA in plants, specifically in glutamate, glutamine, aspartate, asparagine, valine, and alanine (Zhu et al., 2018). Finally, nitrate accumulation is also observed in response to stress including excessive fertilization, drought, or reduced sunlight as a result of increased uptake or decreased utilization for protein synthesis (Nicholson, 2007). As a result, it is important to monitor situations which may be stressful for the plant or lead to stunted growth as they may alter the AA profile or contribute to accumulation of NCCs which may be damaging for the horse.

Amino Acid Profile. Considering fractions within CP, such as NPN, do not relate to protein content that the horse can use, the AA profile is a more accurate indicator of protein quality and quantity. While individual AA requirements have not been established for the horse, with the exception of lysine, some AA profiles have been reported in feeds (Table 1.1). These gross values differ across feed types. For example, pelleted grain and alfalfa consistently have the highest lysine concentrations ≥ 0.73% DM while other forages such timothy hay and pasture vegetation from a CSG-mixture observed the lowest lysine values at ≤ 0.29% DM. However, discrepancies have been observed with pasture vegetation from the current study observing lysine values ≥ 0.72% DM with a CSG-mixture pasture having the highest values at 1.25% DM. It is likely these discrepancies van be attributed to other factors that influence CP including forage species, maturity, and nitrogen fertilization.

Additionally, regardless of the differences in gross values, relative proportions of AAs are quite comparable across all feed types (Table 1.2). For example, with the exception of timothy hay, lysine values range from 11.2 to 14.0% of total essential AAs

in all feeds. Similarly, threonine ranges from 8.3 to 11.4% and methionine ranges from 1.9 to 4.5% of total essential AAs. Relative proportions of AAs are equally important to gross AA values in order to prevent the occurrence of limiting AAs which may impair protein synthesis in the horse.

PHYSIOLOGICAL RESPONSE OF HORSES IN RESPONSE TO FEED

While the nutritive values and feed components are important to evaluate when formulating a ration for your horse, how these nutrients are digested, absorbed, and utilized within the horse are more important. As a result, it is crucial to not only understand what we feed horses but the physiological relevance of the nutritive values.

Glucose and Insulin Response in Horses

These differences in the nutritive values of the forages are important as they can elicit different responses in horses following the consumption of a meal. A key physiological response in horses, which is often used to evaluate metabolic conditions, is the glucose and insulin response. This value is often associated with the NSC concentration of a feed. However, other factors have been evaluated for their role in the glucose and insulin response. But to better understand this concept, it is crucial to understand the process in which insulin is digested, absorbed and utilized by the body.

Digestion and Absorption. When a horse consumes a meal, it goes through mechanical breakdown in the mouth before going down the esophagus into a simple stomach. Following the stomach, carbohydrates will either be hydrolyzed in the small intestine or continue to the hindgut where they will be fermented; whether a carbohydrate will be hydrolyzed or fermented depends on the linkage of the sugar molecules. Carbohydrates which are linked via α-1,4-linkages, including starch and maltose, go

through enzymatic hydrolysis in the small intestine while β-1,4 linked molecules, such as mucilages, pectins, and fructans, will be fermented in the hindgut.

To begin carbohydrate hydrolysis, enzymes such as α-amylase, α-glucosidases, and β-galactosidase are secreted. As the carbohydrates proceed down the small intestine, they are ultimately hydrolyzed to yield fructose, glucose, and galactose to be absorbed in the small intestine. Once they become mono- or disaccharides, the carbohydrates can be absorbed by a Na+ glucose cotransporter type I (SGLT1) to enter the enterocytes. When the carbohydrates accumulate within the enterocytes, they will then be transported down the concentration gradient via a facilitative glucose transporter (GLUT1) into systemic circulation. As a result, absorption of carbohydrates in the small intestine will contribute to the plasma glucose response of a horse after a meal.

Blood glucose following a meal will then be sensed by the pancreas which will release insulin from the β-cells to stimulate glucose uptake by the organs including skeletal muscle, the liver, and adipose tissue. Upon uptake, glucose will be converted to glucose-6-phosphate to either be utilized in glycolysis or for glycogen synthesis. Additionally, glucose can contribute to the glycerol backbone in triglycerides.

Carbohydrates that are not absorbed in the small intestine will reach the hindgut where they are fermented by microbes to yield volatile fatty acids including acetate, propionate, and butyrate. However, fermentation of certain carbohydrates such as starch and fructans are believed to result in increased lactic-acid producing bacteria including amylolytic and saccharolytic bacteria. As a result, excess starch and fructans reaching the hindgut can lead to reduced hindgut pH and are implicated in metabolic problems such as

laminitis in horses (Van Eps and Pollitt, 2006). As a result, it is important to monitor starch and fructan intake, especially in horses who are at risk for metabolic diseases.

Responses in Various Feed Types. While digestion and absorption of carbohydrates follows a straightforward process, the glucose and insulin response of a horse after a meal can vary based on the characteristics of the feed. Horses consume a wide range of feed, most often in the form of forage or concentrates. These different feeds can impact of the glucose and insulin response of horses. As a result, research has been performed to evaluate feed characteristics that may contribute to these differences.

In a study performed by Gordon et al. (2008), both feed form and consumption time are believed to influence the glycemic and insulinemic response of horses. The results of this study indicate that oval shaped feed extended the consumption time which led to increased average glucose and insulin responses following a meal whereas the pelleted feed was consumed more quickly and resulted in the lowest average glucose and insulin values. Additionally, meal size is believed to play a role as a study performed by Gordon et al. (2007) evaluated low NSC (9.4%) or high NSC (18.1%) diets which were fed at a rate to provide 0.3 g/kg BW NSC per meal. When feeding these diets to have equal NSC content, glucose responses were similar yet the higher NSC feed resulted in a substantially lower insulin response. These results suggest that a smaller meal size is capable of eliciting a diminished insulin response. Feed processing is also capable of altering the glucose and insulin response as determined by Nielsen et al. (2010). When comparing uniquely processed feeds, the pelleted steam-processed corn appeared to have the highest glycemic response while the cracked corn had the lowest.

The protein and amino acid (AA) concentration of a meal may also impact the glucose and insulin response post-consumption. In a study done by Calbet and MacLean (2002) in humans, it was determined that combined administration of carbohydrates and protein exhibited a synergistic release of insulin. Similarly, a study performed by Stull and Rodiek (1988), evaluating the glucose and insulin response in horses consuming alfalfa pellets in comparison to an alfalfa corn diet, corn only diet, and a corn and corn oil diet, concluded that the insulin response can be influenced by multiple facets of the dietary content including carbohydrates, amino acids, and fat. However, in a study performed by Sticker et al. (1995), horses consuming a control versus restricted protein diet exhibited no differences in their glucose and insulin response following feed consumption. Due to these conflicting results, the role of protein and insulin values needs to be evaluated further in horses.

While research has evaluated the glucose and insulin response of horses consuming concentrates, forage constitutes the main portion of the diet and needs to be evaluated more extensively. In research performed in hay, Shepherd et al. (2012) evaluated the effect of a low- versus high-NSC hay on overweight geldings over a 28-d period. While horses consuming the high (18% DM) NSC hay had a greater insulin concentration on day 7 compared to horses fed a moderate (12% DM) NSC hay, differences in NSC was not capable of sustaining different insulin responses over the 28-day period. In recent research, the insulin and glucose concentrations of horses consuming teff compared to ryegrass hay was evaluated (Askins et al., 2017). While horses consuming teff hay had decreased intake, resting insulin and glucose concentrations did not differ over a 10-d period. However, evaluating long-term

responses and the glucose and insulin response post-consumption would be valuable in future research. It is important to note that the results from both of these studies indicates that differences observed in different types of hay are not capable of eliciting altered responses in horses.

Forage management is another factor that needs to be considered when grazing horses. Siciliano et al. (2017) evaluated the relationship between sward height and the glycemic and insulinemic response of horses grazing tall fescue. This study observed no differences in the glycemic response but an attenuated insulinemic response in horses grazing the shorter sward height of 15 cm when compared to the taller sward height between 30 and 40 cm. In further research evaluating pasture management techniques on the glucose and insulin response, Williams et al. (2017) evaluated the impact of rotational versus continuous grazing on the glucose and insulin dynamics of grazing horses. In this study, it was determined that the grazing system did not impact the glucose and insulin response in horses with these results likely attributed to soluble carbohydrate concentrations lacking physiological relevance to elicit such a change.

Additional research has also compared the glycemic and insulinemic responses of horses consuming pasture-only diets to horses fed an additional concentrate feed (Staniar et al., 2007). In this research, horses consuming a low starch feed (12.8% NSC) had an insulinemic response comparable to horses consuming a pasture-only diet (8.7% NSC) while horses consuming both of these diets observed a decreased insulinemic response when compared to horses consuming a high starch diet (45.7% NSC). Similarly, a study by Richards and Kempton (2016) evaluated the glycemic and insulinemic response of horses consuming warm season pasture (7% NSC), copra meal (11% NSC), extruded

pellet (25.3% NSC), and sweet feed (33.7% NSC). While pasture and copra meal had a significantly lower glycemic response in comparison to extruded pellets and sweet feed, pasture elicited the lowest insulinemic response.

Insulin Resistance. The insulin transduction pathway is comprised of trigger mechanisms which act to send signals throughout the cell in response to blood glucose levels. When carbohydrates are consumed, digested, and absorbed, a rise in the blood glucose concentration is observed. The pancreas senses the elevated blood glucose levels and subsequently releases insulin which binds to the insulin receptor, promoting glucose uptake from the bloodstream and into fat and muscle cells. As a result, the insulin transduction pathway is a key component of maintaining glucose homeostasis.

Unfortunately, as a result of various factors including obesity, genetic predisposition, pregnancy, lactation, and nutritional factors, the cells in the horse's body may lose sensitivity to insulin in what is known as insulin resistance (IR). This disorder is commonly characterized by either hyperinsulinemia or abnormal glycemic or insulinemic responses and either occurs prior to insulin receptor binding or subsequent to insulin binding (Kronfeld et al., 2005; Treiber et al., 2006). Considering IR has been implicated as a risk factor for multiple diseases including laminitis (Geor and Harris, 2009), osteochondrosis (OCD; Ralston, 1996), obesity (Carter et al., 2009; Hoffman et al., 2003), and exertional rhabdomyolysis (Finno et al., 2010), it is important to prevent or manage this condition carefully.

Risk Factors for Insulin Resistance in Horses. Understanding the risk factors associated with insulin resistance is an important way to identify horses who may be at risk for this condition.

Physical characteristics are one of the most common and obvious indicators a horse may have EMS and IR. Specifically, cresty neck is a common phenotype observed in horses with EMS or IR. When the mean neck circumference was measured in a study performed by Frank et al. (2006), this physical characteristic was most closely correlated with AUC for insulin. These results suggest neck enlargement could be a risk factor for IR in horses.

Obesity is another characteristic that is often believed to play a causative role in the onset of IR. Obesity in horses has been linked to IR in two separate theories including (1) adipokines and cytokines playing a role in down-regulating the insulin signaling pathway or (2) lipotoxicity where intracellular lipids accumulate in insulin-sensitive tissue (ie: skeletal muscle). Research conducted by Hoffman et al. (2003) confirmed this association as a decrease in insulin sensitivity was observed in obese horses as they appeared to rely on glucose-mediated disposal of glucose. Similarly, Carter et al. (2009) found a relationship between diet-induced weight gain and decreased insulin sensitivity which ultimately resulted in hyperinsulinemia and hyperleptinemia. The relationship between body condition score (BCS) and body fat percentage with insulin sensitivity has also been evaluated to support this association as Vick et al. (2007) observed a decrease in insulin sensitivity concurrently with an increase in BCS and body fat percentage. As a result, body weight and BCS can often be evaluated to assess obesity and the risk of IR. Additionally, based on these studies, the management or prevention of obesity could be a useful strategy helping reduce the risk of IR and EMS in horses.

While obesity is largely associated with horses consuming more energy than they expend, there is also a genetic factor involved. In research performed by McCue et al.

(2015), not only did environmental risk factors play a role in metabolic trait variation, but there was variability in the risk of alleles, allele penetrance, gene-by-environment, and gene-by-gene interaction. The differences across breed phenotypes likely reflects breed-by-breed allele differences as well as their relative importance. While genetic predisposition and breed phenotype need to be considered in the development of obesity, the diet plays a crucial part as well.

Protein and Amino Acids with Horses

While evaluating the glucose and insulin response is beneficial, understanding protein and amino acids in horse nutrition is an overlooked subject in the horse industry. Proteins, and amino acids specifically, are crucial as they play many physiological roles, not only limited to protein synthesis, but also involving neurotransmitter and hormone synthesis as well as in conditions associated to vitamin and mineral utilization.

Digestion and Absorption. As proteins are consumed by the horse, the first key step of digestion begins in the stomach. Here, peptide bonds are cleaved by HCl and pepsin secreted by the parietal and chief cells, respectively. At this point, partially digested proteins reach the duodenum of the small intestine where they are further broken down by pancreatic enzymes, including chymotrypsin, trypsin, elastase, carboxypeptidase, and enterokinase. During this process, polypeptides are broken down into AAs, dipeptides, or tripeptides which can either be absorbed by enterocytes or further hydrolyzed by brush-border enzymes until they can be absorbed. Following absorption, AAs can be used by enterocytes or taken up and enter the blood stream of the animal as they are delivered to various tissues and utilized for protein synthesis. While

NPN can also be absorbed, it cannot be incorporated into protein but rather is excreted in the urine.

Any proteins or AAs that escape absorption in the small intestine, reach the hindgut in which they are fermented. During this process, peptides and AAs can be taken up by microbes and utilized for microbial protein synthesis, metabolized to VFA's, or synthesize AAs. However, considering the hindgut succeeds the portion of the GI tract responsible for protein absorption, the horse cannot absorb or utilize the protein products of fermentation.

Plasma Amino Acid Levels. As previously mentioned, AAs will be absorbed in the small intestine following a meal before they enter the blood stream. These values are often measured in terms of plasma AA and a sharp rise and peak are observed before the plasma AA concentrations decrease and reach a nadir. Where these peaks and valleys occur in plasma AAs is dependent on numerous factors including feed type and meal schedule.

In a study performed by Johnson and Hart (1974), plasma free AA concentrations were evaluated either following a fast or following consumption of a pelleted ration. This study determined plasma AA values peaked 2 h post-consumption with essential AA peaking at 132% basal levels before a rapid decline while nonessential AA peaked and plateaued at 120% of basal values until 8 h post-consumption. Approximately 18 to 30 h following a fast, values would decrease to near basal levels once again. However, following 30 h, a rise in essential AAs are observed until plasma concentrations reach 130% basal levels. These increases are likely a result of AA release into the plasma from the tissues in addition to decreased muscle protein anabolism.

In addition to variations in plasma AA in response to feeding versus fasting, alterations in the plasma AA response can be a result of meal schedule. When a complete pelleted diet was fed to horses in 1- or 2-meal feeding schedules, peak plasma free AA were observed at 5 h and 3 h, respectively. This study suggests plasma free AA take longer to peak when the horse is fed one meal a day in comparison to horses fed multiple meals.

Additional research has evaluated feed type by comparing a hay-only versus a hay-grain diet (Graham-Thiers and Bowen, 2011). During this study, horses on the hay-grain diet observed peaks 1 h post-consumption while horses consuming timothy orchardgrass hay observed peaks between 2 and 3 h post-consumption. These results suggest horses receiving concentrates observe peak plasma AA concentrations sooner than horses on a complete feed or a hay-only diet.

Amino Acid Requirements. Plasma AA concentrations can be used to evaluate the absorption of AAs in the feed. The goal when feeding protein to a horse is to provide adequate amounts and appropriate ratios of amino acids to meet the horse's nutritional needs. When the amino acid requirements are met, the animal can synthesize tissues, hormones, and enzymes, as well as repair tissues as needed (NRC, 2007). Unfortunately, individual amino acid requirements have yet to be determined for the horses, with CP content used to balance a horse's diet based on the nitrogen (N) content of the feed. However, the CP calculation does not accurately reflect the amount of protein the horse can digest and use as not all nitrogen is present in feed is a component of proteins. Instead, the amino acid concentrations of feeds should be analyzed and balanced in order for horses to properly utilize them.

However, since these AA requirements have yet to be established for the horse, protein is commonly fed in excess, in the form of grain or concentrates, to ensure requirements are being met (Graham-Thiers and Kronfeld, 2005). Unfortunately, there are numerous negative implications associated with excess protein or nitrogen content of a feed including environmental implications and metabolic costs to the horse. Environmental implications include water contamination and decreased air quality due to the release of nitrogen in the form of urea which is converted to ammonia. Additionally, excess protein consumption is associated with increased metabolic energy cost associated with excess N excretion in urine as well as increased water intake to accommodate excretion.

However, determining the protein requirements is not a simple matter as there are numerous moving parts to be considered including age, gender, workload, or physiological parameters such as the reproductive status.

In exercising horses, Wickens et al. (2003) evaluated horses fed varying amounts of dietary protein at 677 g/d, 790 g/d, 903 g/d, 1,016 g/d, and 1,129 g/d. Horses consuming the diets with 1,016 g/d showed improved N retention which resulted in a recommendation of 1.9 to 2.1 g CP/kg BW/d for moderately exercised horses. However, when Wickens et al. (2005) evaluated protein requirements based on minimizing 3-methyl-histidine (3MH) concentrations, a component indicative of muscle breakdown, the data predicted the CP requirement to be at 954 g/d. Further research has shown conflicting results as Patterson et al. (1985) evaluated horses at different work intensities with data reporting a requirement of 410 mg digestible protein/kg BW/day.

In a separate study performed by Orton et al. (1985), results suggested exercising horses had improved dietary protein efficiency. This was revealed when 2-year-old horses had similar average daily gains (ADG) when consuming either 1.2 or 2.4 g DP/kg BW/d. However, exercising horses still observed greater ADG than horses at maintenance consuming diets with similar protein. These results suggest protein content did not influence ADG but rather exercise played a role.

In growing horses, a study by Mastellar et al. (2016) evaluated yearling horses fed graded amounts of lysine. Altering levels of lysine is valuable as it is currently believed to be the most limiting amino acids in horses. This study did not reveal a breakpoint when comparing varying lysine levels to whole body protein synthesis which suggests there might've been a more limiting amino acid to underscore the results. Further research by Tanner et al. (2014) compared a commercial CP diet (4.1 g CP/kg BW/day) compared to the recommended CP diet (3.1 g CP/kg BW/day), according to the NRC. The results from this study indicated lower whole-body protein synthesis in weanlings fed the recommended CP diet to suggest an amino acid was limiting.

Limiting Amino Acids. A limiting amino acid, which refers to an amino acid which cannot be synthesized by the body in sufficient quantities to meet the requirements, can be restricting in the diet. Understanding which amino acid is limiting is important as protein quality is directly dependent on the content of the most limiting amino acid relative to the horse's needs. This means an amino acid imbalance can still occur if a second limiting amino acid is added to a diet when the first limiting amino acid is deficient. As a result, if a diet does not provide adequate quantities of essential amino acids, protein synthesis cannot proceed beyond the rate at which those amino acids are

available. Additionally, the necessary amino acids must be available at the same time; which is why they are known as limiting amino acids (NRC, 2007).

To accommodate for inadequate quantities of essential AAs while preventing excess, ideally horses would consume precise amounts of AAs to meet the dietary requirements of the first limiting AAs, rather than increasing the CP content as a whole. This is a method that has already been implemented in other monogastric feeding systems, specifically swine and poultry. One study performed by Kerr et al. (1995) evaluated high- and low-CP diets with or without lysine, tryptophan, and threonine supplementation. This study found that proper AA supplementation could correct the reduced pig performance and carcass muscle commonly observed with decreased CP content of the diet. A separate study (Hsu et al., 1998) evaluated methionine supplementation in laying hens with similar findings; laying hens fed low protein with methionine supplementation had similar egg production and feed conversion when compared to laying hens on high-protein diets. These studies confirm that decreased CP with proper AA supplementation can still meet the protein and AA requirements of the animal.

Currently, lysine and threonine are believed to be the most limiting amino acids to the horse. To evaluate the impact of supplementing these amino acids, Graham-Thiers and Kronfeld (2005) set out to evaluate horses supplemented with lysine, threonine. This study took place over a 14-week period with horses participating in light exercise for the duration of the trial. The results from this study indicate improved muscle mass maintenance, lower body condition score, no change in body weight, greater creatinine, and lower 3-methyl-histidine (3MH) and plasma urea nitrogen (PUN) concentrations in

horses receiving supplementation. Overall, supplementing the limiting amino acids might be necessary to performance horses to minimize excess protein consumption while maximizing muscle mass.

However, Mastellar et al. (2016) further evaluated threonine supplementation on whole-body protein synthesis in mature horses. These results were different than the results previously reported as threonine supplementation did not increase whole-body protein synthesis and was determined to not be a limiting amino acid for these horses. However, supplementing with threonine did lead to altered plasma concentrations of other amino acids, to demonstrate the ability of supplementing an amino acid to influence the metabolism of other amino acids. As a result, while supplementing amino acids may be useful to performance horses for improving protein efficiency while minimizing excess protein, supplementation needs to be precise and careful and more research needs to be performed on individual amino acid supplementation before specific recommendations can be made.

The contradictory results of lysine and threonine versus threonine-only supplementation on improved muscle mass could be the result of limiting amino acids. While the most limiting amino acid is not known for horses, it is currently believed to be lysine. Supplementation of threonine without lysine would be ineffective if enough lysine was not present and available in the diet. Additionally, the alteration in the amino acid concentrations could be a result of amino acids competing for binding sites for absorption in the small intestine.

Protein and Feed Types. In addition to supplementation, the format of consumption is one of the most important variables to consider when determining if the

diet will meet the protein requirements of the horse. Forage, in the form of hay or pasture, as well as concentrates, fed as extruded, pelleted, or grain rations, are the two most common forms of feed consumption in the horse. However, these different feed types have many unique characteristics which impact overall protein utilization including amino acid profiles, digestibility, availability, intake, and digestive rate. Fortunately, research has been performed to evaluate the relationship between feeds types and proteins in the horse.

Research performed by Gibbs et al. (1988) evaluated the digestion of horses consuming coastal bermudagrass (CB; 11.7% CP), low-protein alfalfa (LA; 15.0% CP), or high-protein alfalfa (HA; 18.1% CP). This study observed a trend toward greater relative prececal digestibility observed in horses consuming HA in comparison to horses consuming the other forages. Additionally, the higher N retention as a percentage of intake was higher in horses consuming HA to indicate a larger proportion of N in HA was absorbed in the small intestine as amino acids rather than ammonia in the hindgut. These results suggest increased protein content, indicated by higher CP, as well improved quality, indicated by decreased fiber associated with improved digestibility, are key factors in selecting hay for performance horses. Overall, increasing forage quality has demonstrated increased AA availability and digestion in the horse diet.

Additionally, a study was performed by Connysson et al. (2006) to evaluate the capability of forage-only diets to meet the protein requirements of Standardbred horses in racing conditions. This study fed horses to meet or exceed the current CP requirements set by the NRC (1989) by feeding either a high protein (HP; 16.6% CP) or low protein (RP; 12.5% CP) diet. This study determined horses consuming HP forage had increased

water intake, increased excretion of N in urine and feces, and increased plasma urea levels to suggest greater N metabolism. Despite consuming higher energy feed, horses consuming the HP forage did not exhibit weight gain. This may be contributed to the higher need for energy in intermediary metabolism of excess N to urea and the subsequent excretion of N compounds in urine. Additionally, the acid-base balance was altered with the HP forage causing acidosis and challenging the fluid balance in horses at rest. Decreased urine pH was observed within the first day with water intake for protein excretion increases within the first day, while a 2 day period was required to 'wash out' excessive nitrogen in the horse. These results demonstrate the disadvantages to horses consuming excess protein, even on a forage-only diet, and indicate HP diets are an unnecessary challenge to performance horses.

However, while there are numerous negative implications associated with excess protein, concentrates and grains can be fed to horses to improve N use efficiency by increasing protein availability and digestibility in comparison to forages such as hay. This was demonstrated in a study comparing hay versus a hay-grain diet where horses consuming grain had earlier and more drastic spikes in the blood AA pool following feed consumption, to demonstrate increased AA digestibility and availability in comparison to the hay-only diet (Graham-Thiers and Bowen, 2011). However, while concentrates are shown to have increased protein digestibility and availability, increased urea N in the hay-grain diet suggests excess AA were being consumed (Graham-Thiers and Bowen, 2011).

To further evaluate the negative implications of excess protein in horses consuming concentrates, Miller and Lawrence (1988) evaluated different dietary protein

levels, of either 12.9% CP (control) or 18.5% CP (high-protein), in exercising horses. This study revealed minimal differences between horses consuming different levels of protein in the diet with key differences observed including higher urea-N levels, and lower alanine levels in horses consuming high protein. While this specific study did not uncover metabolic evidence regarding the detrimental impact of excess protein, the researchers acknowledged further implications to acknowledge. These implications included the inability of protein to act as an optimal energy source as urea synthesis and excretion require energy, which can compete with other energy needs of the equine athlete.

These studies have revealed information regarding the different feed types. Specifically, high quality diets, such as concentrates, will have improved digestibility and availability of amino acids. When forages are considered, the higher quality forages, indicated by greater CP and lower fiber concentrations, offer greater availability and utilization of amino acids via improved prececal digestibility. However, while most of these studies have observed N losses with increasing protein diets, more work has to be done to identify the requirement for horses to make sure they are not receiving excess. While concentrates and high quality forage are the best way to ensure the horse is meeting their protein requirements, it is important not to feed diets too high in protein which will result in metabolic costs to the horse.

Excess Protein Consumption. Protein fed at a rate which exceeds amino acid requirements will not be used to synthesize proteins as excess protein is not stored. While little research has been done on excess protein consumption in horses, feeding protein in excess of requirements has been a common practice in the horse industry (Harper, 2009).

Horses are provided rations formulated with higher than the recommended dietary protein levels to ensure all essential amino acid requirements are being met. However this practice does not ensure that there is not a deficiency in any single, limiting amino acids (NRC, 2007).

When excess levels of protein are fed, the nitrogen is stripped away and the carbon skeletons of the excess amino acids are oxidized for energy or stored as fat and glycogen. However, the amino nitrogen can be transferred to another carbon skeleton to make a non-essential amino acid otherwise it must be excreted as ammonia or urea. An increase in nitrogen excretion is capable of directly impacting the horse, as observed in other species (Funaba et al., 1996; Kim et al., 2011) through increased urine output; which may also lead to increased water requirements. The increase of urine output associated with excess protein can decrease the retention of Ca (Delimaris, 2013; Heaney, 2002; Roughead, 2003); this may lead to problems including a decrease in bone mineralization as reviewed by Barzel and Massey (1998) and Delimaris (2013), and potentially lower bone density which can be especially problematic in exercising horses (Myburgh et al., 1990). Graham-Thiers et al. (1999, 2000) also concluded excess protein could result in a lower blood pH in exercising horses at rest or completing sprints as a result of an acid-base balance interference.

Nitrogen excretion, in the form of urea, can also indirectly impact horse health through the production of ammonia. When urea comes in contact with urease, an enzyme commonly found in feces, they react to form ammonia. Due to the high level of urease activity in feces, the conversion of urea to ammonia occurs rapidly after excretion. A study in Japan found the inhalation of high concentrations of ammonia is detrimental to

the respiratory health of horses with signs of severe nasal discharge, swelling and irregular distribution of tracheal epithelium and edema of submucosa, and loss of nasal cilia (Katayama et al. 1995). High ammonia rates don't only impact horses, agricultural workers exposed to ammonia contamination have exhibited headaches, eye irritation, and naseau (Schiffman et al., 2005). Additionally, Greger and Koneswaran (2010) found that 25% of workers at swine concentrated animal feeding operations reported at least one of the following respiratory symptoms: asthma, bronchitis, acute respiratory distress syndrome, and organic dust toxicity syndrome. Respiratory health can be affected by ammonia due to its ability to react with other compounds to form particulate matter (PM) with a diameter of 2.5 microns or less (PM2.5). This specific classification of PM is concerning because the small size of the particles allows them to penetrate deep into the lungs.

Environmental concerns are another factor to be considered when feeding excess protein. Estimations by FAO (2001) hold agriculture responsible for over 75% of ammonia emissions, with livestock contributing to the majority of ammonia production. Three environmental concerns associated with excess nitrogen excretion include accumulation of nutrients in the soil, eutrophication in water, and global warming. First, excess nutrients in manure have the capability to leach into the soil. Sensitive crops including tomatoes, cucumbers, conifers, and fruit cultures can be damaged by over-fertilization as a result of ammonia deposition (Van der Eerden et al., 1998). The deposition of ammonia on soils with a low buffering capacity also has the capability of causing soil acidification and basic cation depletion (Sheffield, 2012). Additionally, the leaching of nitrogen from manure is causing water nitrate to exceed tolerable levels. This

form of nutrient enrichment can lead to harmful algal growth and the eutrophication can lead to a decline in aquatic species (Sharpley et al., 1994). Ammonia volatilization is also a concern as nitrate in the soil can be changed to ammonia gas (NH_3), an emission effecting global warming (McCrory and Hobbs, 2001). In respect to greenhouse gases (GHG) responsible for trapping heat in the atmosphere, the global contribution to GHG emissions by the animal sector as a result of manure and urine is: 35 to 40% for CH_4 and 65% for N_2O (Steinfield et al., 2006). These concerns bring up issues regarding the sustainability of natural resources. In horses, reducing the crude protein content of feed while supplying adequate amounts of amino acids would be one way to minimize these concerns. Additionally, waste management, specifically related to nitrogen excretion as a result of excess protein, is being measured in livestock and continues to be a concern.

Protein Deficiency. While there are concerns regarding excess protein in livestock diets, there are also detrimental impacts resulting from protein deficiency; adequate amounts of protein and amino acids are needed for maintenance and growth of the horse. While protein deficiency rarely occurs as an isolated condition, low protein intake can lead to lack of growth, poor musculature, poor hair quality, reduced feed intake, and reduced hoof growth in horses (NRC, 1989). Additional physical signs of protein deficiency include weight loss in adult horses, fetal loss in pregnant mares, low milk production in lactating mares, and a loss of muscle in sedentary or exercising mature horses (NRC, 2007). It is obvious both excess protein and protein deficiency have damaging effects on horses, so it is important we find a balance by determining a ration based on the amino acid profile rather than CP.

Unfortunately, determining an amino acid requirement is not a simple task. Limitations on studies done in the whole horse can be traced to high costs associated with feeding and housing horses as well as confounding factors associated with other organs and body systems. Additionally, horse euthanasia is highly controlled due to the conflicting role of the horse between livestock and a companion animal.

EQUINE MUSCLE AND PROTEIN SYNTHESIS

Equine Skeletal Muscle

While there are limitations to evaluating AA requirements in horses using feeding trials, equine skeletal muscle in cell culture provides a unique alternative to evaluate AAs in the horse diet. Skeletal muscle is an important component of the horse as it represents the largest tissue mass, constituting approximately 50% of the horse's BW (Gunn, 1987). Skeletal muscle is a composite structure of muscle cells, organized networks of nerves and blood vessels, and an extracellular connective-tissue matrix (Huard et al., 2002). This framework functions to produce joint movement and to support the regeneration process that occurs after injury.

The basic structural element of the skeletal muscle is muscle fibers, also known as myofibers; a syncytium derived from the fusion of multiple myogenic precursor cells, or myoblasts. These myoblasts fuse to form long, cylindrical, multinucleated myotubes that exhibit central nucleation. A myonuclei shift from the central to subsarcolemmal position allows the muscle cells to then be classified as myofibers. The sarcoplasm is the cytoplasm of a myofiber unit and contains a cellular matrix and organelles. Additionally, each myofiber is surrounded by a connective tissue layer known as the endomysium while a bundle of these myofibers is surrounded by the perimysium. Each myofiber unit

also has a sarcolemma, or a plasma membrane surrounding the myofiber as well as a basal lamina or basement membrane; which is a 100 to 200-nm-thick external connective tissue layer comprised of proteins including collagen, fibronectin, laminin, and many glycoproteins (Gates and Huard, 2005).

The capacity of skeletal muscle to regenerate after injury was documented primarily in German literature in the mid-19th century (Scharner and Zammit, 2011). However, it wasn't until Alexander Mauro (1961) detected the satellite cell that cellular regenerative potential was fully recognized. These mononucleated cells lie under or are embedded in the basal lamina of the myofiber while remaining in contact with the plasma membrane (Soeta et al., 1998). Satellite cells are an important component of the muscle; while they are quiescent during most of adult life, they can become activated following injury, stress, or exercise and play a key role in the muscle regeneration process (Baquero-Perez et al., 2012; Huard et al., 2002). After activation, satellite cells divide and differentiate to contribute to muscle hypertrophy by adding new nuclei to preexisting fibers as satellite cell progeny, also known as myoblasts. Myoblasts then undergo repeated mitosis in order to fuse into pre-existing myotubes or form new myotubes, which is similar to embryological development of muscles (Baquero-Perez et al., 2012).

The rate of growth during the neonatal period surpasses all other stages of postnatal life, and skeletal muscle is responsible for the majority of the mass increase. The neonatal period is classified as the time when an animal is dependent on their caretakers for nutrition, which can also be described as up until weaning; this stage coincides with the attainment of full biochemical and functional maturity of the skeletal muscle. Growth can occur when the rate of protein synthesis exceeds the rate of protein

degradation; which can be attributed to the accelerated rates of protein synthesis in neonates alongside the rapid accumulation of muscle nuclei (Davis et al., 2009). The enhanced activation of insulin and amino acid signaling components in skeletal muscle contributes to the rapid gain of skeletal muscle protein mass in neonates.

By using satellite cells derived from equine skeletal muscle in cell culture, a controlled environment, including specific media contents, timing, and order of applications, is created to analyze the direct impact of numerous variables on the muscle.

Importance of Muscle Cells in Culture

Skeletal muscle can be isolated and studied *in vitro* to evaluate various aspects of skeletal muscle biology (Baquero-Perez et al. 2012). Previous studies have developed a method to isolate equine satellite cells from equine skeletal muscle (Greene and Raub, 1992). Considering protein is the largest nonwater component of muscle, constituting approximately 65% of the dry mass of equine skeletal muscle (Badiani et al., 1997), evaluating satellite cells isolated from skeletal muscle in culture is a valuable technique to study amino acids in the horse.

While satellite cells are more prominent in young muscle, they are present in mature skeletal muscle in a mitotically quiescent state (Moss and Leblond, 1971; Schultz et al., 1978). Satellite cells play a key functional role in postnatal muscle growth and development (Moss and Leblond et al., 1971) as well as regeneration of injured muscle tissue (Heslop et al., 2000; Shiaffino et al., 1976). By studying these cells in culture, under controlled conditions, it is possible to determine external and internal factors that may play a role in enhancing growth and development in young horses as well as enhanced muscle recovery in injured horses.

Unfortunately, there are some setbacks with using muscle cells in culture. While research done by Allen et al. (1979) suggests myoblasts in culture can fuse into multinucleated myotubes accumulating muscle-specific protein, differentiation may be incomplete as myotubes express embryonic or neonatal isoforms of contractile proteins (Minty et al., 1982; Whalen, 1980) and enzymes (Iannaccone et al., 1982; Perriard et al., 1982). While these concerns need to be recognized, the ability to culture and isolate satellite cells allows us to analyze intrinsic properties of satellite cells in the absence of changing environmental factors.

Protein Synthesis

Hypertrophy and atrophy of muscle mass is determined by relative rates of muscle protein synthesis; which can be impacted by nutritional (Svanberg et al., 1996; Yoshizawa et al. 1998) and hormonal (Umpleby and Russell-Jones, 1996) status, physical activity or inactivity (Ferrando et al., 1996), age (Forbes and Reina, 1970), and pharmacological therapies (Diaz et al., 2015). While skeletal muscle has a vital mechanical role, skeletal muscle protein has the capacity to store energy and amino acids to be used during injury, starvation, or disease to fulfill an important metabolic role (Rennie and Tipton, 2000). However, the ability of the muscle to store energy and amino acids is dependent on the dynamic state of muscle protein mass. As a result, having the capability to measure muscle protein synthesis is an important factor to determine the functionality of the muscle both mechanically and metabolically.

Previous methods used to measure muscle protein synthesis have centered around measuring the incorporation of a radioactively labeled tracers into proteins. While these methodologies can be very successful, expensive equipment and specialized techniques

complicate the use of these approaches. High costs can be associated with the use and disposal of a radioactive tracer, administrative and record-keeping costs, as well as equipment such as scintillation counters. Radioactive tracers may also lack the sensitivity required to detect protein synthesis differences between cells within the same tissue.

Recent work has developed a nonradioactive technique known as surface sensing of translation (SUnSET; Schmidt et al., 2009). In order to detect puromycin incorporation into nascent peptide chains, antibiotic puromycin (a structural analog of tyrosyl-tRNA) and anti-puromycin antibodies are used (Nathans, 1964; Schmidt et al., 2009). The incorporation of puromycin is a measurement of puromycin-conjugated peptides and accurately reflects the rate of protein synthesis (Nakano and Hara, 1979; Schmidt et al., 2009). Most of the current research regarding this study has been done in the skeletal muscle due to high interest in identifying the molecular metabolism relating to skeletal muscle protein synthesis (Goodman et al., 2011). Research performed by Goodman et al. (2011) was the first study to use this nonradioactive technique to measure changes in protein synthesis *ex vivo* and *in vivo* in whole tissues via western blots and at a single-cell level via immunohistochemistry. These techniques were validated in this study and demonstrate the ability to visualize and quantify protein synthesis as well as eliminate the need for radioactivity in either tissues or animals.

Table 1.1. Amino acid (AA) concentrations (% DM) in feed types used in previous equine studies.

Feed Type	Arg[1]	Cys	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Tyr	Val	Source
	% DM												
Alfalfa Pasture	0.89	0.22	0.43	0.95	1.57	1.17	0.33	1.02	0.81	0.28	0.57	1.16	Current Study
Cool-season Grass Pasture	1.07	0.25	0.43	1.01	1.75	1.25	0.41	1.18	0.92	0.3	0.61	1.28	
Teff Pasture	0.57	0.14	0.23	0.58	1.02	0.72	0.24	0.65	0.51	0.15	0.33	0.74	
Bermudagrass Hay	0.49	-	0.23	0.32	0.56	0.47	0.09	0.37	0.31	-	0.15	0.40	Strakova et al., 2013
Pasture Vegetation	0.33	-	0.15	0.26	0.44	0.29	0.05	0.24	0.26	-	0.14	0.32	
Timothy Hay	0.36	0.03	0.12	0.24	0.41	0.24	0.07	0.27	0.28	-	0.14	0.31	Woodward et al., 2011
Alfalfa Hay (Mid-bloom)	0.72	0.04	0.36	0.53	0.86	0.73	0.11	0.55	0.62	-	0.28	0.66	
Alfalfa Hay (Early-bloom)	0.91	0.04	0.4	0.6	0.97	0.76	0.12	0.63	0.69	-	0.31	0.73	
Alfalfa Hay (Early-bud)	0.96	0.04	0.46	0.68	1.18	0.89	0.15	0.76	0.77	-	0.37	0.89	
Bermudagrass Hay	0.46	0.11	0.15	0.37	0.75	0.49	0.15	0.44	0.4	0.12	0.28	0.49	DePew et al., 1994
Pelleted Grain	0.95	0.23	0.35	0.58	1.18	0.78	0.23	0.72	0.58	0.16	0.48	0.73	
Timothy/Orchardgrass Hay	0.34	-	0.14	0.31	0.57	0.41	0.13	0.4	0.33	-	-	0.41	Graham-Thiers and Bowen, 2011
Grain (Omolene 100; Purina)	0.66	-	0.26	0.34	0.77	0.51	0.18	0.47	0.35	-	-	0.49	

[1]Amino acid abbreviations: arginine (arg), cysteine (cys), histidine (his), isoleucine (ile), leucine (leu), methionine (met), phenylalanine (phe), threonine (thr), tryptophan (trp), tyrosine (tyr), and valine (val).

Table 1.2. Relative proportions of amino acids (AAs) in relation to the total essential amino acid (EAA) composition as reported in previous studies evaluating common feed types fed to horses.

Feed Type	Total EAA	Arg[1]	Cys	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Tyr	Val	Source
	% DM	% Total EAA												
Alfalfa Pasture	9.4	9.5	2.3	4.6	10.1	16.7	12.4	3.5	10.9	8.6	3.0	6.1	12.3	Current Study
CSG Pasture	10.5	10.2	2.4	4.1	9.7	16.7	12.0	3.9	11.3	8.8	2.9	5.8	12.2	
Teff Pasture	5.9	9.7	2.4	3.9	9.9	17.3	12.2	4.1	11.1	8.7	2.6	5.6	12.6	
Bermudagrass Hay	3.4	14.5	-	6.6	9.4	16.6	14.0	2.7	10.9	9.1	-	4.4	11.8	Strakova et al., 2013
Pasture Vegetation	2.5	13.4	-	6.0	10.4	17.7	11.8	2.0	9.8	10.5	-	5.7	12.8	
Timothy Hay	2.5	14.6	1.2	4.9	9.7	16.6	9.7	2.8	10.9	11.3	-	5.7	12.6	Woodward et al., 2011
Alfalfa Hay (Mid-bloom)	5.5	13.2	0.7	6.6	9.7	15.8	13.4	2.0	10.1	11.4	-	5.1	12.1	
Alfalfa Hay (Early-bloom)	6.2	14.8	0.6	6.5	9.7	15.7	12.3	1.9	10.2	11.2	-	5.0	11.9	
Alfalfa Hay (Early-bud)	7.2	13.4	0.6	6.4	9.5	16.5	12.4	2.1	10.6	10.8	-	5.2	12.4	
Bermudagrass Hay	4.2	10.9	2.6	3.6	8.8	17.8	11.6	3.6	10.5	9.5	2.9	6.7	11.6	DePew et al., 1994
Pelleted Grain	7.0	13.6	3.3	5.0	8.3	16.9	11.2	3.3	10.3	8.3	2.3	6.9	10.5	
Timothy/Orchardgrass Hay	3.0	11.2	-	4.6	10.2	18.8	13.5	4.3	13.2	10.9	-	-	13.5	Graham-Thiers and Bowen, 2011
Grain (Omolene 100; Purina)	4.0	16.4	-	6.5	8.4	19.1	12.7	4.5	11.7	8.7	-	-	12.2	

[1]Amino acid abbreviations: arginine (arg), cysteine (cys), histidine (his), isoleucine (ile), leucine (leu), methionine (met), phenylalanine (phe), threonine (thr), tryptophan (trp), tyrosine (tyr), and valine (val).

LITERATURE CITED

Allen, E., C. Sheaffer, and K. Martinson. 2012. Yield and persistence of cool-season grasses under horse grazing. Agron. J. 104(6):1741-1746.

Allen, E., C. Sheaffer, and K. Martinson. 2013. Forage nutritive value and preference of cool-season grasses under horse grazing. Agron. J. 105(3): 679-684.

Allen, R.E., M.H. Stromer, D.E. Goll, and R.M. Robson. 1979. Accumulation of myosin, actin, tropomyosin, and α–actinin in culture muscle cells. Dev. Biol. 69: 655-660.

Andrews, F.M., and J.A. Nadeau. 1999. Clinical syndromes of gastric ulceration in foals and mature horses. Equine Vet. J. Suppl. 29:81-85.

Askins, M.J., A.G. Palkovic, K.A. Leppo, G.C. Jones, and J.C. Gill. 2017. Effect of feeding teff hay on dry matter intake, digestible energy intake and resting insulin/glucose concentration in horses. J. Eq. Vet. Sci. 52: 45.

Badiani, A., N. Nanni, P.P. Gatta, B. Tolomelli, and M. Manfredini. 1997. Nutrient profile of horsemeat. J. Food Comp. Anal. 10: 254-269.

Balde, A.T., J.H. Vendersall, R.A. Erdman, J.B. Reeves III, and B.P. Glenn. 1993. Effect of stage of maturity of alfalfa and orchardgrass on in situ dry matter and crude protein degradability and amino acid composition. Anim. Feed Sci. Technol. 44:29-43.

Baquero-Pérez, B., S.V. Kichioudi, R.K. Nelli, and K. Chang. 2012. A simplified but robust method for the isolation of avian and mammalian muscle satellite cells. BMC Cell Biol. 13: 16.

Barzel, U.S., and L.K. Massey. 1998. Excess dietary protein can adversely affect bone. J. Nutr. 128: 1051-1053.

Borgia L.A., S.J. Valberg, M.E. McCue, K. Watts, and J.D. Pagan. 2011. Glycemic and insulinemic responses to feeding hay with different nonstructural carbohydrate content in control and Polysaccharide Storage Myopathy-affected horses. J. Anim. Physiol. Anim. Nutr. (Berl). 95(6): 798-807.

Bruhn, J.C., and J.C. Oliver. 1978. Effect of storage on tocopherol and carotene concentrations in alfalfa hay. J. Dairy Sci. 61: 980-982.

Buxton, D.R. 1996. Quality-related characteristics of forages as influenced by plant environment and agronomic factors. Anim. Feed Sci. Tech. 59: 37-49.

Calbet, J.A.L., and D.A. MacLean. 2002. Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans. J. Nutr. 132: 2174-2182.

Campbell-Thompson, M.L., and A.M. Merritt. 1987. Effect of ranitidine on gastric acid secretion in young male horses. Am. J. Vet. Res. 48:1511-1515.

Carter, R.A., L.J. McCutcheon, L.A. George, T.L. Smith, N. Frank, and R.J. Geor. 2009. Effects of diet-induced weight gain on insulin sensitivity and plasma hormone and lipid concentrations in horses. Am. J. Vet. Res. 70: 1250-1258.

Catalano, D.N., C.C. Sheaffer, A.M. Grev, M.L. Schultz, and K.L. Martinson. 2015. Forage nutritive value, yield and preference of legumes under horse grazing. J. Equine Vet. Sci. 35:435.

Cerling, T.E., Y. Wang, and J. Quade. 1993. Expansion of C4 ecosystems as an indicator of global ecological change in the late Miocene. Nature. 361: 344±345.

Connysson, M., S. Muhonen, J.E. Lindberg, B. Essen-Gustavsson, G. Nyman, K. Nostell, and A. Jansson. 2006. Effects on exercise response, fluid and acid-base balance of protein intake from forage-only diets in Standardbred horses. Equine Vet J. Suppl. 36: 648-653.

Crozier, J.A., V.G. Allen, N.E. Jack, J.P. Fontenot, and M.A. Cochran. 1997. Digestion, apparent mineral absorption, and voluntary intake by horses fed alfalfa, tall fescue, and Caucasian bluestem. J. Anim. Sci. 75:1651-1658.

Davis, T.A. and M.L. Fiorotto. 2009. Regulation of muscle growth in neonates. Curr. Opin. Clin. Nutr. Metab. Care. 12(1): 78-85.

DeBoer, M.L., C.C. Sheaffer, A.M. Grev, D.N. Catalano, M.S. Wells, M.R. Hathaway, and K.L. Martinson. 2017. Yield, nutritive value, and preference of annual warm-season grasses grazed by horses. Agron. Journal. 109(5):1-13.

DeBoer, M.L., M.R. Hathaway, K.J. Kuhle, P.S.D. Weber, A.S. Reiter, C.C. Sheaffer, M.S. Wells, and K.L. Martinson. 2018. Glucose and insulin response of horses grazing alfalfa, perennial cool-season grass, and teff across seasons. J. Equine Vet. Sci. Accepted Manuscript.

Delimaris, I. 2013. Adverse effects associated with protein intake above the recommended dietary allowance for adults. ISRN Nutr. 2013: 1-6.

Diaz, E.C., D.N. Herndon, C. Porter, L.S. Sidossis, O.E. Suman, and E. Borsheim. 2015. Effects of pharmacological interventions on muscle protein synthesis and breakdown in recovery from burns. Burns. 41(4): 649-657.

Elizalde, J.C., N.R. Merchen, and D.B. Faulkner. 1999. Fractionation of fiber and crude protein in fresh forages during the spring growth. J. Anim. Sci. 77:476-484.

FAO. 2001. Global estimates of gaseous emissions of NH3, NO and N2O from agricultural land, International Fertilizer Industry Association. Rome.

Ferrando, A.A., H.W. Lane, C.A. Stuart, J. Davis-Street, and R.R. Wolfe. 1996. Prolonged bed rest decreases skeletal muscle and whole body protein synthesis. Am. J. Phys. 270(4): E627-E633

Finno, C.J. E. McKenzie, S.J. Valberg, and J. Pagan. 2010. Effect of fitness on glucose, insulin, and cortisol responses to diets varying in starch and fat content in Thoroughbred horses with recurrent exertional rhabdomyolysis. Equine Vet. J. Suppl. 38: 323-328.

Fonnesbeck, P.V., M.M. Garcia de Hernandez, J.M. Kaykay, and M.Y. Saiady. 1986. Estimating yield and nutrient losses due to rainfall on field-drying alfalfa hay. Anim. Feed. Sci Technol. 16:7-15.

Forbes G.B. and J.C. Reina. 1970. Adult lean body mass declines with age: some longitudinal observations. Metabolism. 19: 653–663.

Frank, N., S.B. Elliot, L.E. Brandt, and D.H. Keisler. 2006. Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. J. Am. Vet. Med. Assoc. 228: 1383-1390.

Frank N. 2009. Insulin resistance and equine metabolic syndrome. *In* Robinson, N.E., and K.A. Sprayberry (eds.), Current Therapy in Equine Medicine. p. 727-740. Elsevier Publications.

Fry, J. and B. Huang. 2004. Applied turf grass science and physiology. Wiley, New York, NY.

Funaba, M., M. Hashimoto, C. Yamanaka, Y. Shimogori, T. Iriki, S. Ohshima, and M. Abe. 1996. Effects of a high-protein diet on mineral metabolism and struvite activity product in clinically normal cats. Am. J. Vet. Res. 57: 1726-1732.

Gates, C., and J. Huard. 2005. Management of skeletal muscle injuries in military personnel. Oper. Techn, Sports Med. 13: 247-256.

Geor, R.J., and P.A. Harris. 2009. Dietary management of obesity and insulin resistance: countering risk for laminitis. Vet. Clin. Equine. 25: 51-65.

Gibbs, P.G., G.D. Potter, G.T. Schelling, J.L. Krieder, and C.L. Boyd. 1988. Digestion of hay protein in different segments of the equine digestive tract. J. Anim. Sci. 66:400-406.

Goodman, C.A., D.M. Mabrey, J.W. Frey, M.H. Miu, E.K. Schmidt, P. Pierre, and T.A. Hornberger. 2011. Novel insights into the regulation of skeletal muscle protein synthesis as revealed by a new nonradioactive in vivo technique. FASEB J. 25: 1028-1039.

Gordon, M.E., M.L. Jerina, C.L. King, K.E. Davison, J.K. Young, and R.H. Raub. 2007. The effects of nonstructural carbohydrate content and feeding rate on glucose and insulin

Gordon, M.E., M.L. Jerina, R.H. Raub, K.E. Davison, and J.K. Young. 2008. The effects of feed form on consumption time and glucose and insulin response to a concentrate meal in equine. J. Eq. Vet. Sci. 28(5): 289-294.

Graham-Thiers, P.M., and L.K. Bowen. 2011. Effect of protein source on nitrogen balance and plasma amino acids in exercising horses. J. Anim. Sci. 89:729-735.

Graham-Thiers, P.M. D.S. Kronfield, and K.A. Kline. 1999. Dietary protein influences acid-base responses to repeated sprints. Eq. Vet. J. Suppl. 30: 463-467.

Graham-Thiers, P.M., D.S. Kronfield,, K.A. Kline, D.J. Sklan, and P.A. Harris. 2000. Protein status of exercising Arabian horses fed diets containing 14% or 7.5% crude protein fortified with lysine and threonine. J. Equine Vet. Sci. 20: 516-521.

Graham-Thiers, P.M., and D.S. Kronfeld. 2005. Amino acid supplementation improves muscle mass in aged and young horses. J. Anim. Sci. 83(12):2783-2788.

Greene, E.A. and R.H. Raub. 1992. Procedures for harvesting satellite cells from equine skeletal muscle. J. Equine Vet. Sci. 12: 33-35.

Greger M., and G. Koneswaran. 2010. The public health impacts of concentrated animal feeding operations on local communities. Fam. Community Health. 33: 11-20.

Grev, A.M., C.C. Sheaffer, M.L. DeBoer, D.N. Catalano, and K.L. Martinson. 2017. Preference, yield, and forage nutritive value of annual grasses under horse grazing. Agron. J. 109: 1561-1572.

Gunn, H.M. 1987. Muscle, bone, and fat proportions and muscle distribution of thoroughbreds and other horses. p. 253-264. *In*: Gillespie JR, Robinson NE, editors. Equine exercise physiology 2: proceedings of the second international conference on equine exercise physiology. Davis, CA: ICEEP Publications.

Hare, P.D., and W.A. Cress. 1997. Metabolic implications of stress-induced proline accumulation in plants. J. Plant Growth Regul. 21(2):79-102.

Harkin, J.M. 1973. Lignin. P. 323 – 373. In G.E. Butler and R.W. Bailey (ed.) Chemistry and biochemistry of herbage. Academic Press. New York, NY.

Heaney, R.P. 2002. Protein and calcium: antagonists or synergists? The Am. J. of Clin. Nutr. 75: 609-610.

Heslop, L., J.E. Morgan, and T.A. Partridge. 2000. Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. J. Cell Sci. 113: 2299-2308.

Hoffman, R.M., R.C. Boston, D. Stefanovski, D.S. Kronfeld, and P.A. Harris. 2003. Obesity and diet affect glucose dynamics and insulin sensitivity in thoroughbred geldings. J. Anim. Sci. 81: 2333-2342.

Holt, D.A., and A.R. Hilst. 1969. Daily variation in carbohydrate content of selected forage crops. Agron. J. 61(2):239-242.

Hsu, J, C. Lin, and P.W. Chiou. 1998. Effects of ambient temperature and methionine supplementation of a low protein diet on the performance of laying hens. Anim. Feed Sci. Technol. 74:289-299.

Huard, J., Y. Li, and F.H. Fu. 2002. Muscle injuries and repair: current trends in research. J. Bone Joint Surg. Am. 84(5): 822-830.

Iannaccone, S.T., B. Nagy, and F.J. Samaha. 1982. Partial biochemical maturation of aneurally cultured human skeletal muscle. Neurology. 32: 846-851.

Johnson, C.R., B.A. Reiling, P. Mislevy, and M.B. Hall. 2001. Effects of nitrogen fertilization and harvest data on yield, digestibility, fiber, and protein fractions of tropical grasses. J. Anim. Sci. 79(9):2439-2448.

Johnson, R.J., and J.W. Hart. 1974. Influence of feeding and fasting on plasma free amino acids in the equine. J. Anim. Sci. 38(4): 790-794.

Katayama Y, Oikawa M, Yoshihara T, Kuwano A, Hobo S. 1995. Clinico-Pathological Effects of Atmospheric Ammonia Exposure on Horses. J. Equine Vet. Sci. 6: 99-104.

Kerr, B.J., F.K. McKeith, and R.A. Easter. 1995. Effect on performance and carcass characteristics of nursery to finisher pigs fed reduced crude protein, amino acid-supplemented diets. J. Anim. Sci. 73:433-440.

Kim, K.I., S. Lee, and R. Choue. 2011. Metabolic responses to high protein diet in Korean elite bodybuilders with high-intensity resistance exercise. J. of the Int. Soc. Of Sports Nutr. 8.

Kronfeld, D.S., K.H. Treiber, T.M. Hess, and R.C. Boston. 2005. Insulin resistance in the horse: definition, detection and dietetics. J. Anim. Sci. 83: E22-E31.

LaCasha, P.A., H.A. Brady, V.G. Allen, C.R. Richardson, and K.R. Pond. 1999. Voluntary intake, digestibility, and subsequent selection of Matua bromegrass, coastal bermudagrass, and alfalfa hays by yearling horses. J. Anim. Sci. 77:2766-2773.

Lechtenberg, V.L., D.A. Holt, and H.W. Youngberg. 1971. Diurnal variation in nonstructural carbohydrates in vitro digestibility, and leaf to stem ratio of alfalfa. Agron. J. 63(5):719-724.

Longland, A.C., and B.M. Byrd. 2006. Pasture nonstructural carbohydrates and equine laminitis. J. Nutr. 136(7): 20995-21025.

Martinson, K.L., M.S. Wells, and C.C. Sheaffer. 2016. Horse preference, forage yield, and species persistence of 12 perennial cool-season perennial grass mixtures under horse grazing. J. Equine Vet. Sci. 36: 19-25.

Martinson, K., M. Hathaway, J.H. Wilson, B. Gilkerson, P.R. Peterson, and R.D. Vecchio. 2006. University of Minnesota Horse Owner Survey: Building an Equine Extension Program. Journal of Extension. 44(6): 6RIB4.

Mastellar, S.L, A. Moffet, P. Harris, and K.L. Urschel. 2016. Effects of threonine supplementation on whole-body protein synthesis and plasma metabolites in growing and mature horses. The Vet. J. 126: 93-100.

Mauro, A. 1961, Satellite cell of skeletal muscle fibers. J. Biophys. Biochem. Cytol. 9: 492-495.

McCormick, J.S., R.M. Sulc, D.J. Barker, and J.E. Beuerlein. 2006. Yield and nutritive value of autumn-seeded winter-hardy and winter-sensitive annual forages. Crop Sci. 46(5): 1981.

McCowan, S., M. Brummer, S. Hayes, G. Olson, S.R. Smith, and L. Lawrence. 2012. Acceptability of teff hay by horses. J. Equine Vet. Sci. 32:327-331.

McCrory, D.F., and P.J. Hobbs. 2001. Additives to reduce ammonia and odor emissions from livestock wastes. A review. J. Environ. Qual. 30: 345-355.

McCue, M.E., R.J. Geor, and N. Schultz. 2015. Metabolic syndrome: a complex disease influenced by genetics and the environment. J. Equine Vet. Sci. 35: 367-375.

Miller, P.A., and L.M. Lawrence. 1988. The effect of dietary protein level on exercising horses. J. Anim. Sci. 66: 2185-2192.

Minty, A.J., S. Alonso, M. Caravatti, and M.E. Buckingham. 1982. A fetal skeletal muscle actin mRNA in the mouse and its identity with cardiac actin mRNA. Cell 30: 185-192.

Moore, J.E. and G.O. Mott. 1973. Structural inhibitors of quality tropical grasses. *In* Matches, A.G. (ed.), Anti-quality components of forages. p. 53-98. Madison: Crop Science Society of America.

Moore, K.J., and H.G. Jung. 2001. Lignin and Fiber Digestion. J. Range Managem. 54(4):420-430.

Moore, K.J., T.A. White, R.L Hintz, P.K. Patrick, and E.C. Brummer. 2004. Sequential grazing of cool- and warm-season pastures. Agron. J. 96: 1103-1111.

Moss, F.P. and C.P. Leblond. 1971. Satellite cells as a source of nuclei in muscles of growing rats. Anat. Rec. 170: 421-436.

Mowat, D.N., R.S. Fulkerson, W.E. Tossell, and J.E. Winch. 1965. The in vitro digestibility and protein content of leaf and stem portions of forages. Can. J. Plant Sci. 45:321-331.

Murray, M.J. 1992. Gastric ulceration in horses: 91 cases (1987 – 1990). J. Am. Vet. Med. Ass. 201:117-120.

Murray, M.J., and G.F. Schusser. 1993. Measurement of 24-h gastric pH using an indwelling pH electrode in hroses unfed, fed, and treated with ranitidine. 25(5): 417-421.

Mus, F., M.B. Crook, K. Garcia, A.Garcia Costas, B.A. Geddes, E.D. Kouri, P. Paramasivan, M. Ryu, G.E.D. Oldroyd, P.S. Poole, M.K. Udvardi, C.A. Voigt, J. Ané, and J.W. Peters. 2016. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Appl. Environ. Microbiol. 82(13): 3698-3710.

Myburgh, K.H., J. Hutchins, A.B. Fataar, S.F. Hough, and T.D. Noakes. 1990. Low bone density is an etiologic factor for stress fractures in athletes. Ann. Intern. Med. 113: 754-759.

NAHMS, 1998. Equine'98, Part I: Baseline reference of 1998 equine health and management. National Animal Health Monitoring System, USDA: APHIS: VS, Fort Collins, USA, 1998.

Nakano, K. and H. Hara. 1979. Measurement of the protein-synthetic activity in vivo of various tissues in rats by using [^{3}H]puromycin. Biochem J. 184: 663-668.

Nathans, D. 1964. Puromycin inhibition of protein synthesis: incorporation of puromycin into peptide chains. Proc. Natl. Acad. Sci. U.S.A. 51: 585-592.

Nicholson, S.S. 2007. Nitrate and nitrite accumulating plants. In: R.C. Gupta, editor, Veterinary toxicology, basic clinical principles. Elsevier. Amsterdam, the Netherlands. p. 876 – 879.

Nielsen, B.D., C.I. O'Connor-Robison, H.S. Spooner, and J. Shelton. 2010. Glycemic and insulinemic responses are affected by age of horse and method of feed processing. J. Eq. Vet. Sci/ 30(5): 249-258

NRC. 1989. Nutrient Requirements of Horses. 5th ed. The National Academy Press, Washington D.C.

NRC. 2007. Nutrient Requirements of Horses. 6th ed. The National Academy Press, Washington, D.C.

Orton, R.K., I.D. Hume, and R.A. Leng. 1985. Effects of level of dietary protein and exercise on growth rates of horses. Equine Veterinary Journal. 17: 381-385.

Palmonari, A., M. Fustini, G. Canestrari, E. Grili, and A. Formigoni. 2014. Influence of maturity on alfalfa hay nutritional fractions and indigestible fiber content. J. Dairy Sci. 97:7729-7734.

Patterson, P.H., C.N. Coon, and I.M. Hughes. 1985. Protein requirements of mature working horses. J. Anim. Sci. 61: 187-196.

Perriard, J.-C., U.B. Rosenberg, T. Walliman, H.M. Eppenberger, and M. Caravatti. 1982. The switching of creatine kinase gene expression during myogenesis. *In* Pearson, M.L. and H.F. Epstein (eds.), Muscle Development: Molecular and Cellular Control. p. 237-245. Cold Spring Harbor Laboratory, New York.

Ralston, S.L. 1996. Hyperglycemica/hyperinsulinemia after feeding a meal of grain to young horses with osteochondritis dissecans (OCD) lesions. Pferdeheilkunde. 12: 320-322.

Reid, R.L., G.A. Jung, and W.V. Thayne. 1988. Relationships between nutritive quality and fiber components of cool season and warm season forages: a retrospective study. J. Anim. Sci. 66: 1275-1291.

Rennie, M.J. and K.D. Tipton. 2000. Protein and amino acid metabolism during and after exercise and the effects of nutrition. Annu. Rev. Nutr. 20: 457-483.

Richards, N., and T.J. Kempton. 2016. The post feeding glycaemic and insulin response to copra meal in horses. Anim. Feed Sci. Technol. 211:100-108.

Riesterer, J.L., M.D. Casler, D.J. Undersander, and D.K. Combs. 2000. Seasonal yield distribution of cool-season grasses following winter defoliation. Agron. J. 92: 974-980.

Rodiek, A.V., and C.L. Stull. 2007. Glycemic index of ten common horse feeds. J. Equine Vet. Sci. 27(5):205-212.

Rodiek, A.V., and B.E. Jones. 2012. Voluntary intake of four hay types by horses. J. Equine Vet. Sci. 32(9):579-583.

Rotz, C.A., and R.E. Muck. 1994. Changes in forage quality during harvest and storage. G.C. Fahey, M. Collins, D.R. Mertens, L.E. Moser (Eds.), Forage Quality, Evaluation, and Utilization, American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wisc., USA (1994), pp. 828-868.

Roughead, Z.K. 2003. Is the interaction between dietary protein and calcium destructive or constructive for bone?: Summary. J. Nutr. 133: 866S-869S.

Sage, R., and D. Kubien. 2003. Quo vadis C4? An ecophysiological perspective on global change and the future of C4 plants. Photosynth. Res. 77: 209–225.

Salon, C., N. Munier-Jolain, G. Duc, A. Voisin, D. Grandgirard, A. Larmure, R.J.N. Emery, and B. Ney. 2001. Grain legume seed fillin in relation to nitrogen acquisition: A review and prospects with particular reference to pea. Agronomie. 21:539-552.

Scharner, J., and P.S. Zammit. 2011. The muscle satellite cell at 50: the formative years. Skeletal muscle 1:28.

Schiaffino, S., S. Pierobon Bormioli, and M. Aloisi. 1976. The fate of newly formed satellite cells during compensatory muscle hypertrophy. Virchows Arch. B. Cell Pathol. 21: 113-118.

Schiffman S., C. Studwell, L. Landerman, K. Berman, and J. Sundy. 2005. Symptomatic effects of exposure to diluted air sampled from a swine confinement atmosphere on healthy human subjects. Environ. Health Persp. 113: 567-576.

Schmidt, E.K., G. Clavarino, M. Ceppi, and P. Pierre. 2009. SUnSET, a nonradioactive method to monitor protein synthesis. Nature Methods. 6(4): 275-277.

Schultz, E., M.C. Gibson, T. Champion. 1978. Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. J. Exp. Zool. 206: 451-456.

Sharpley, A., S. Chapra, R. Wedepohl, J. Sim. T. Daniel, K. Reddy. 1994. Managing agricultural phosphorus for protection of surface waters: Issues and options. J. Environ. Qual. 23: 437-451.

Sheffield, R. 2012. Atmospheric Ammonia: Understand Its Effects. Louisiana State University Extension.

Shepherd, M.L., R.S. Pleasant, M.V. Crisman, S.R. Were, S.C. Milton, and W.S. Swecker Jr. 2012. Effects of high and moderate non-structural carbohydrate hay on insulin, glucose, triglyceride, and leptin concentrations in overweight Arabian geldings. J. Anim. Phys. Anim. Nutr. 96: 428-435.

Siciliano, P.D., J.C. Gill, and M.A. Bowman. 2017. Effect of sward height on pasture nonstructural carbohydrate concentrations and blood glucose/insulin profiles in grazing horses. J. Equine Vet. Sci. 57:29-34.

Sleugh, B., K.J. Moore, J.R. George, and E.C. Brummer. 2000. Binary legume-grass mixtures improve forage yield, quality, and seasonal distribution. Agron. J. 92:24-29.

Soeta, C. K. Yamanouchi, T. Hasegawa, N. Ishida, H. Mukoyama, H. Tojo, and C. Tachi. 1998. Isolation of satellite cells from equine skeletal muscle. J. Equine Vet. Sci. 9(3): 97-100.

Staniar, W.B., T.A. Cubitt, L.A. George, P.A. Harris, and R.J. Geor. 2007. Glucose and insulin response to different dietary energy sources in thoroughbred broodmares grazing cool season pasture. Liv. Sci. 111: 164-171.

Staniar, W.B., J.R. Bussard, N.M. Repard, M.H. Hall, and A.O. Burk. 2010. Voluntary intake and digestibility of teff hay fed to horses. J. Anim. Sci. 88:3296-3303.

Steinfeld, H., P. Gerber, T. Wassenaar, V. Castel, M. Rosales, and C. de Haan. 2006. Livestock's long shadow: Environmental issues and options. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Sticker, L.S., D.L. Thompson Jr., L.D. Bunting, J.M. Fernandez, and C.L. DePew. 1995. Dietary protein and(or) energy restriction in mares: plasma glucose, insulin, nonesterified fatty acid, and urea nitrogen responses to feeding, glucose, and epinephrine. J. Anim. Sci. 73: 136-144.

Stull, C.L. and A.V. Rodiek. 1988. Responses of blood glucose, insulin, and cortisol concentrations to common equine diets. J. Nutr. 118: 206-213.

Svanberg, E., L.S. Jefferson. K. Lundholm, and S.R. Kimball. 1997. Postprandial stimulation of muscle protein synthesis is independent of changes in insulin Am. J. Physiol. 272: E841-E847.

Tanner, S.L., A.L. Wagner, R.N. Digianantonio. P.A. Harris, J.T. Sylvester, and K.L. Urschel. 2014. Dietary crude protein intake influences rates of whole-body protein synthesis in weanling horses. Vet. J. 202: 236-243.

Trieber, K.H., D.S. Kronfeld, and R.J. Geor. 2006. Insulin resistance in equids: possible role in laminitis. J. Nutr. 136: 2094S-2098S.

Umpleby, A.M. and D.L. Russell-Jones. 1996. The hormonal control of protein metabolism. Bailliere Clin. Endoc. 10(4): 551-570.

Van der Eerden, L.J.M., P.H.B de Visser, and C.J. Van Dijk. 1998. Rise of damage to crops in the direct neighborhood of ammonia sources. Environ. Pollut. 102(51): 49-53.

Van Eps, A.W., and C.C. Pollitt. 2006. Equine laminitis induced with oligofructose. Equine Vet. J. 38(3):203-208.

Vick, M.M., A.A. Adams, B.A. Murphy, D.R. Sessions, D.W. Horohov, R.F. Cook, B.J. Shelton, and B.P. Fitzgerald. 2007. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J. Anim. Sci. 85: 1144-1155.

Weir, W.C., L.G. Jones, and J.H. Meyer. 1960. Effect of cutting interval and stage of maturity on the digestibility and yield of alfalfa. J. Anim. Sci. 19(1): 5-19.

Weyenberg, S.V., J. Sales, and G.P.J. Janssens. 2006. Passage rate of digesta through the equine gastrointestinal tract: a review. Livestock Sci. 99(1): 3 – 12.

Whalen, R.G. 1980. Contractile protein isozymes in muscle development: The embryonic phenotype. *In* Pette, D. (ed.), Plasticity of Muscle. p. 177-191. Walter de Gruyter, Berlin.

Wickens, C.L., J. Moore, J. Shelle, C. Skelly, and N.L. Trottier. 2003. Effect of exercise on dietary protein requirement of the Arabian horse. In: Proc. Eq. Nutr, Physiol. Society, East Lansing, MI. 18:129-131.

Wickens, C.L., J. Moore, C. Wolf, C. Skelley, and N. Trottier. 2005. 3-methylhistidine as a response criterium to estimate dietary protein requirement of the exercising horse. In: Proc. Eq. Nutr. Physiol. Society. 19:205.

Willard, J.G., J.C. Willard, S.A. Wolfram, and J.P. Baker. 1977. Effect of diet on cecal pH and feeding behavior of horses. J. Anim. Sci. 45:87-93.

Williams, C.A., D.S. Kronfeld, W.B. Staniar, and P.A. Harris. 2001. Plasma glucose and insulin responses of thoroughbred mares fed a meal high in starch and sugar or fat and fiber. J. Anim. Sci. 79:2196-2201.

Williams C.A., L.B. Kenny, and A.O. Burk. 2017. Effects of grazing system and season on glucose and insulin dynamics of the grazing horse. J. Eq. Vet. Sci. 52: 87.

Woodward, A.D., B.D. Nielsen, J. Liesman, T. Lavin, and N.L. Trottier. 2011. Protein quality and utilization of timothy, oat-supplemented timothy, and alfalfa at differing harvest maturities in exercised Arabian horses. J. Anim. Sci. 89:4081-4092.

Yoshizawa, F., S.R. Kimball, T.C. Vary, and L.S. Jefferson. 1998. Effect of dietary protein on translation initiation in rat skeletal muscle and liver. Am. J. Physiol. 275: E814-E820.

Zhu, G., H. Xiao, Q. Guo, Z. Zhang, J. Zhao, and D. Yang. 2018. Effects of cadmium stress on growth and amino acid metabolism in two compositae plants. Ecotoxicol. Environ. Saf. 158:300-308.

Chapter 2

Plasma amino acid concentrations of horses grazing alfalfa, cool-season perennial grass, and teff

Michelle L. DeBoer[a], Krishona L. Martinson[a], Kerry J. Kuhle[b], Craig C. Sheaffer[c], Marcia R. Hathaway[a]

[a]Department of Animal Science, University of Minnesota, St. Paul, MN

[b]Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN

[c]Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN

SUMMARY

The impact of forage species on plasma amino acid (AA) concentrations of grazing horses (*Equus caballus* L.) is unknown. The objectives of this study were to determine the impact of different forage species on plasma AA concentrations and protein synthesis. Research was conducted in July in St. Paul, MN. Alfalfa (*Medicago sativa L.*), mixed perennial cool-season grasses (CSGs), and teff (*Eragrostis tef* [Zucc.] Trotter) pastures were grazed by six horses randomly assigned to one of three forage types in a replicated Latin-square design. Horses had access to pasture each day. Jugular venous blood samples were collected from each horse prior to being turned out (0 h) and then at 2 and 4 h post-turnout. Corresponding forage samples were taken by hand harvest and analyzed for AA concentrations. Equine muscle satellite cell cultures were treated with sera from grazing horses to assess *de novo* protein synthesis. Data were analyzed using PROC

MIXED in SAS with $P \leq 0.05$ being significant and trends identified at $P \leq 0.10$. When evaluating forage, AA were generally lowest in teff and highest in CSG ($P \leq 0.05$). Significant differences in threonine concentration in the plasma were observed; there was no effect on *de novo* protein synthesis of cultured equine myotubes treated with plasma obtained from the grazing horses ($P \geq 0.20$). As a result, although there were significant differences in forage AA content only plasma threonine concentration was different at 4 h with no effect on protein synthesis of cultured equine satellite cells.

INTRODUCTION

Protein in the diet of the horse is commonly referred to as crude protein (CP); an estimate based on the nitrogen content of the feed. However, AAs are a more accurate predictor of the protein quality and play a crucial role in the body as a constituent of proteins. Unfortunately, with the exception of lysine, AA requirements have yet to be established for the horse (NRC, 2007). Further work is needed since limiting AAs can reduce the rate of protein synthesis. Lysine, methionine and threonine are believed to be the most limiting AA for the horse (NRC, 2007). However, AAs fed to exceed the animals' immediate requirement can also be detrimental as they are not utilized or stored but rather metabolized and the nitrogen is excreted as urea. This is a metabolically expensive process and can lead to potential environmental issues (Bott et al., 2016)

Evaluating the AA profile and overall protein quality of a feed is important when trying to meet the AA requirements of the horse while minimizing excessive AA consumption. While research has evaluated protein quality and utilization in hay (Woodward et al., 2011; Gibbs et al., 1988) and concentrates (Graham-Thiers et al., 2011), little work has evaluated AA utilization in horses grazing pasture (Straková et al.,

2013). However, pasture is a primary component of the equine diet with a survey conducted of Minnesota horse owners finding 87% of respondents with horses have access to pasture (Martinson et al., 2006). The frequency of horses with access to pasture demonstrates the importance of evaluating AA utilization in grazing horses.

While the AA profile is important when evaluating protein quality, foregut bioavailability of a feed is equally important. As a monogastric, hindgut fermenter, the small intestine is the site where protein is digested and the resultant AAs are absorbed in the horse. Although most of the protein in forages is fermented in the hindgut, foregut availability of nutrients improves when protein content increases and fiber content decreases (Gibbs et al., 1988).

However, the CP and fiber concentrations vary across forage species due to differences in plant structures and unique photosynthetic pathways. Specifically, warm-season grasses, such as teff, accumulate higher concentrations of cell wall components as they utilize the C_4 photosynthetic pathway (Wilson et al., 1994); which results in higher fiber values and consequently decreased nutrients such as CP (Reid et al., 1988). In comparison, alfalfa has higher CP values and lower fiber values when compared to grasses (Woodward et al., 2011; Gibbs et al., 1988). These unique nutrient profiles can impact the availability and utilization of AA; as a result, the objectives of this study were to determine the impact of alfalfa, CSG, and teff on plasma AA concentrations and *de novo* protein synthesis of grazing horses.

MATERIALS AND METHODS

All experimental procedures were conducted according to those approved by the University of Minnesota Institutional Animal Care and Use Committee.

Horse Management

Six mares (24 ± 2 yr) were grazed. Horse bodyweight was 494 ± 53 kg with a BCS of 5 ± 1 (Henneke et al., 1983). Horses had *ad libitum* access to water throughout the study and when not grazing, horses were housed in a dry lot and fed a mixed hay containing equal parts alfalfa, CSG and teff at approximately 2.5% bodyweight (BW) split evenly between two daily feedings. Horses were also fed a ration balancer (Enrich Plus Ration Balancing Horse Feed, Purina, St. Louis, MO) at 0.1% BW at 1700 h each day to ensure all vitamin and mineral requirements were met for adult horses at maintenance (NRC, 2007).

Experimental Design and Diets

Horses were randomly assigned to three forage types over three days in a replicated 3 x 3 Latin-square design. Forages consisted of alfalfa, CSG (mixture of orchardgrass (*Dactylis glomerata* L.) and Kentucky bluegrass (*Poa pratensis* L.)), and teff. Alfalfa stands were established in May 2014 in a 0.07 ha pasture and CSG pastures were established in August 2009 in a 0.17 ha pasture. A 0.17 ha teff pasture was established in June 2016 and seeded at a rate of 13.5 kg ha^{-1}. For the entire pasture, the soil was a Waukegan silt loam (fine-silty over skeletal, mixed, superactive, mesic Typic Hapludoll) with a soil pH of 6.6, 18 ppm P, 85 ppm K, and 13 ppm NO_3-N; no fertilization was needed based on soil test results.

Average forage maturity was assessed prior to grazing. Alfalfa maturity was assessed using the mean stage count method (Kalu and Fick, 1983), while maturity for CSG and teff were determined using a scale developed by Moore et al. (1991). Alfalfa

was grazed at the early bud stage with an average maturity of 3. The CSG pasture was grazed at a late vegetative stage, while teff was grazed in the stem elongation phase.

All pastures were mowed to 8 cm three weeks prior to the start of each grazing period to allow for an equal regrowth period. Each pasture was then divided into three equal subplots to allow each horse group (n = 2) access to fresh, un-grazed pasture during the period. Each pasture subplot had sufficient forage available that allowed horses to graze *ad libitum* throughout the 4 h grazing period. Forages were grazed on July 19, 21, and 23, 2016. Prior to the start of the trial, horses received a 24 h hay washout, consisting of equal amounts of each forage species, followed by a 12 h fast. Upon completion of blood collection, horses repeated the hay washout and fast period before switching treatments. This ensured each horse received every treatment, resulting in six observations for each forage type.

Sampling and Analysis

Indwelling catheters were inserted approximately 1 h prior to the start of blood collection using a local anesthetic (2% lidocaine, Lidocaine 20 mg ml^{-1}, VetOne, MWI Animal Health. Boise, ID) blockade. Blood samples were taken at 08:00 (prior to turnout; 0 h) and 2 and 4 h post-turnout, at 10:00 and 12:00 h, respectively. Serum samples were collected in 9-mL serum-separator tubes (8881302015; Covidien, Minneapolis, MN) and left at room temperature for 45 minutes following collection. Plasma samples were collected in 10-mL tubes with an ethylenediaminetetraacetic acid (EDTA) additive (8881311743; Covidien, Minneapolis, MN) and put on ice immediately after collection. Following blood collection, catheter lines were flushed with 10 mL of heparinized saline (1,000 units of heparin 200 mL^{-1} of 0.9% saline). Serum and plasma samples were

separated by centrifugation at 1,200 x g at 4°C for 20 minutes, supernatants were collected, aliquoted and stored at -80°C for later analysis.

Duplicate, representative forage samples were taken from each pasture at 08:00, 10:00, and 12:00 h to correspond to blood samples taken at 0, 2, and 4 h post-turnout. Forage samples were clipped at 8 cm and dried for 24 h at 60º C. After drying, samples were ground through a 6-mm screen in a Wiley mill (Thomas Scientific, Swedesboro, NJ) followed by a 1-mm screen in a Cyclotec (Foss, Eden Prairie, MN). Samples were mixed thoroughly and subsamples were analyzed for forage nutritive value by a commercial forage testing laboratory (Equi-Analytical, Ithaca, NY). Crude protein was calculated as the percentage of N multiplied by 6.25, a method determined by the Association of Official Agricultural Chemists (method 990.03). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were measured using filter bag techniques of ANKOM Technology. Starch concentrations were determined using a glucoamylase enzyme and by measuring dextrose in an automated biochemical analyzer (YSI 2700 select biochemistry analyzer, YSI Incorporated, Yellow Springs, OH). Ethanol soluble carbohydrates (ESC) and water soluble carbohydrates (WSC) were measured using techniques described by Hall et al. (1991). Nonstructural carbohydrates were mathematically estimated by adding WSC plus starch. Equine digestible energy (DE) was calculated using an equation developed by Pagan (1998). Plasma and forage amino acid samples were also analyzed using the AOAC Official method 982.30 (a,b,c) for a proximate analysis (University of Missouri Agricultural Experiment Station Chemical Laboratories, Columbia, MO)

Western Immunoblot of Puromycin Treated Cultures

Recently, a nonradioactive technique known as surface sensing of translation (SUnSET) has been used to assess *de novo* protein synthesis (Schmidt et al., 2009). In order to detect puromycin incorporation into nascent peptide chains, the antibiotic puromycin (a structural analog of tyrosyl-tRNA) and anti-puromycin antibodies were used (Schmidt et al., 2009; Nathans, 1964). The incorporation of puromycin is a measurement of puromycin-conjugated peptides and accurately reflects the initiation of protein synthesis (Schmidt et al., 2009; Nakano and Hara, 1979).

The SUnSET method was used to assess protein synthesis in cultured equine muscle satellite cells. The equine muscle satellite cells were isolated, grown and induced to differentiate into myotubes as previously described (DeBoer et al., 2018). The differentiation medium was replaced with washout media which consisted of the custom formulated Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) which did not contain AA for 6 h after which treatment media was applied. Treatment media consisted of the custom formulated DMEM plus 3% sera; which was obtained from blood collected 4 h post-grazing and pooled by forage from horses grazing either alfalfa, CSG, or teff. After 2 h of treatment media, puromycin (540411; Calbiochem, Billerica, MA, USA) was added to the media in each culture for a final concentration of 5 x 10^{-7} M with the exception of the puromycin negative controls (PURO -); which received only vehicle (custom-formulated DMEM). Cultures were incubated for an additional hour, after which they were collected in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM ethylenediaminetetraacetic, 1% IGEPAL, 1 mM sodium fluoride, 1 mM polymethanesulfonyl fluoride, 1 mM sodium orthovanadate, 104 mM

AEBSF, 80 μM, aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM leupeptin and 1.5 mM Pepstatin). Cell lysate was centrifuged at 10,000 x g for 10 min at 4°C; the supernatant was collected and frozen at -80°C for later analysis.

Protein concentrations were determined with a Micro-BCA protein assay kit (Thermo Fischer Scientific, Waltham, MA) and equivalent amounts of protein (7 μg) of each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on a 4 to 15% mini-protean gradient gel (Bio-Rad; Hercules, CA). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane in high molecular weight protein transfer buffer (242 mM Tris, 58 mM glycine, and 20% v/v methanol). The membrane was blocked in 5% powdered milk in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.5], 150 mM NaCL) with 0.1% Tween 20 (TBST) for 1 h followed by an overnight incubation at 4°C with 1:1,000 anti-puromycin (3RH11; Kerafast, Boston, MA, USA). The membrane was then washed in TBST and incubated for 1 h at 20°C with 1:10,000 horseradish peroxidase conjugated goat anti-mouse IgG (sc-2005; Santa Cruz Biotechnology, Dallas, TX). After TBST washes, membranes were developed by chemiluminescence and viewed by a gel imaging system (LI-COR, Lincoln, NE). Quantification of band densities for each lane were calculated by a computer software program (Image Digits Studio Ver. 3.1). Afterwards, a reversible protein stain (Thermo Fischer Scientific, Waltham, MA) was used to stain the membrane to visually verify equal protein loading of all lanes (Figure 1).

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) as a replicated 3 x 3 Latin-square design. Day and pen were

blocking factors (i.e. rows and columns) in the Latin-square design. Each replicate was the experimental unit for forage. Variables analyzed included DE, CP, NSC, ADF, NDF, and AA values. Data was analyzed using a repeated measures design according to methods used by Littel et al. [1998]. The model included treatment, day, pen, hour, and treatment × hour.

Individual horses within a forage treatment were the experimental unit for plasma AA concentrations. Blood samples taken at hour 0 were included in the model as a baseline covariate. The average blood values were analyzed using a repeated measures design (Littel et al., 1998). The model included treatment, day, pen, hour, and treatment × hour. Means are averaged over the sampling days within the season with the least square means of the MIXED procedure (± SE), and mean separations determined using Tukey's HSD test ($P \leq 0.05$). Statistical significance was set at $P \leq 0.05$ with trends identified at $P \leq 0.10$. Variables analyzed included baseline and average plasma AA values. Relationships between total forage AA, total plasma AA, CP, ADF, and NDF were explored using Pearson Correlation using the CORR procedure of SAS. Statistical significance was set at $P \leq 0.05$.

For the puromyocin blot, each individual culture was considered an experimental unit. Replicate was considered a random effect and treatments were designated as the fixed effect. The variable analyzed was total protein abundance of the treatments for the puromycin western immunoblot. Statistical significance was set at $P \leq 0.05$.

RESULTS

Forage Nutritive Values

All nutritive values differed across forage species during the grazing period (Table 1.1). Alfalfa and CSG had higher DE and CP compared to teff ($P < 0.01$). The NDF and ADF were highest in teff compared to the other species ($P < 0.01$).

Forage Amino Acid Composition

Concentrations of all AA were consistently highest in CSG and lowest in teff; alfalfa tended to be moderate in AA concentrations (Table 1.1). Although alfalfa had the highest CP content, the AA concentrations of alfalfa were never different from CSG. Currently, the most limiting AAs to the horse are believed to be lysine, threonine, and methionine. These AA exhibited a 1.6-fold greater concentration in CSG compared to teff ($P < 0.01$).

Pearson correlation coefficients were utilized to explore the relationship between total forage AA, NDF, ADF and CP. A negative correlation was observed between ADF and total forage AA (0.93; $P \leq 0.01$) and NDF and total forage AA (0.58; $P \leq 0.01$). These results are consistent with other reports in which CP was decreased when fiber content increased (Balde et al., 1993; Elizalde et al. 1999). Additionally, a positive correlation was observed between total forage AA and CP (0.96; $P \leq 0.01$) indicating CP may be used to predict the total forage AA content.

Plasma Amino Acid Composition

No differences in plasma AA concentrations were observed in any of the fasting horses (0 h); therefore, they served as baseline for subsequent comparisons. Differences in plasma threonine concentrations ($P < 0.05$) were observed after 4 h of grazing, with

trends also observed for histidine and methionine ($P < 0.10$; Table 1.2). Horses grazing CSG had higher threonine levels compared to horses grazing teff ($P < 0.05$). Additionally, horses grazing CSG and alfalfa had higher histidine levels than horses grazing teff, and horses grazing CSG had higher methionine levels than horses grazing alfalfa and teff ($P < 0.10$). Plasma AA concentrations were also measured relative to the total AA concentration of the plasma, which was determined by the sum of all essential and nonessential AA (Table 1.3). No differences were observed in AA values relative to total protein, with the exception of threonine at 4 h ($P < 0.05$).

Pearson correlation coefficients were utilized to explore the relationship between total plasma AA, total forage AA, CP, ADF and NDF. A significant but low positive correlation was observed between total forage AA and plasma total AA (0.16; $P < 0.05$). These findings suggest increased forage AA may increase the bioavailability of some AA. In comparison, only a trend was observed between total plasma AA and CP (0.1; $P \leq 0.10$). Cumulatively, these results suggest total forage AA is a better predictor of plasma AA concentrations than CP. Furthermore, a negative correlation was observed between total plasma AA and ADF (0.15; $P \leq 0.05$) with no correlation observed between total plasma AA and NDF.

Puromycin Western Blot

The SUnSET method was used to evaluate the impact of sera, obtained from horses grazing the different forages, on mRNA translation of *de novo* protein synthesis in equine myotube cultures. Band densities of the entire lane, for each of the lanes per treatment, were quantified and averaged to compare alfalfa, CSG, and teff cultures. Alfalfa had an average lane band density of 1,028,000 ± 45,468, in comparison to CSG at

921,750 ± 39,376 and teff at 1,003,250 ± 39,376. No differences in protein synthesis initiation were observed between equine myotube cultures treated with horse sera from horses grazing the different forage species ($P \geq 0.2$). These results indicate that the physiological impact observed for horses grazing different forage species for 4 h has no detectable effect on *de novo* protein synthesis in cultured muscle cells isolated from horses. Equal protein load across the gel and transfer to the blot was verified by reversible protein stain (Figure 1).

DISCUSSION

The major finding of this study is that while teff had consistently lower AA concentrations and a higher fiber content, horses grazing teff only observed lower plasma threonine concentrations with a trend observed for lower plasma histidine and methionine concentrations. Additionally, no differences were observed in *de novo* protein synthesis of equine satellite cells treated for 3 h with sera of horses grazing teff when compared to horses grazing alfalfa and CSG. These results suggest while there were differences in AA concentration and fiber content of the different forages, the digestion of proteins and absorption of AA of the different forages resulted in similar plasma levels.

When consuming a meal, horses exhibit a sharp rise in plasma free AA concentrations two hours post-consumption (Johnson and Hart, 1974). If no additional feedstuff is consumed, this increase is followed by a gradual decline reaching a nadir 12 to 30 h after initiating the fast, followed by a subsequent rise in AA concentration after 30 to 48 h, likely resulting from the degradation of tissues and a release of AA (Johnson and Hart, 1974). As a result, horses need to fast for at least 12 h to reach basal plasma AA

concentrations. In the present study, horses were fasted overnight for at least 12 h but not longer than 15 h prior to pasture turn out.

This practice has been used previously as DePew et al. (1994) observed changes in plasma AA concentrations following an overnight (19 h) fast and post-meal consumption of a pelleted diet. When analyzing the fasting concentrations of indispensable AAs, values were lowest in methionine, near 100 μM, and highest in valine, slightly lower than 300 μM. While this pattern is similar to those observed in the current study, with the lowest fasting concentration observed in methionine and the highest observed in valine, values observed in the current study were much lower, at approximately 22 and 180 μM for methionine and valine, respectively. In exercising horses fasting for 12 h (Graham-Thiers and Bowen, 2011), values were closer to those observed in the current study with methionine having the lowest plasma AA concentrations at 25 μM while valine had the highest values at 143 μM. Other AAs, such as lysine and threonine, had concentrations at 78 and 86 uM, respectively (Graham-Thiers and Bowen, 2011), which is similar to the current study with values around 76 and 80 μM, respectively, for the same AAs. Differences in the results for fasting plasma AA concentrations observed across these studies could be a result of different feedstuffs, meal schedules (Russel et al., 1986), fasting lengths (Johnson and Hart, 1974), horse population, sample collection protocols, or type of sample analysis.

Peak values in plasma AA concentrations following a fast typically occur shortly after meal consumption but are dependent on the feedstuff and meal schedule. Russell et al. (1986) demonstrated peaks in plasma AA concentrations of horses receiving a

complete pelleted ration in either 1 meal or 2 meals each day at 5 and 3 h post-consumption, respectively. In comparison, Graham-Thiers and Bowen (Graham-Thiers and Bowen, 2011) observed peak AA concentrations for horses consuming timothy and orchardgrass hay at 2 to 3 h post-consumption with peaks observed 1 h post-consumption of a hay plus grain diet. Based on these results, it appears horses consuming hay or pasture experience peak AA concentrations 2 to 5 h post-consumption, compared to a grain meal that result in an earlier peak. These findings are consistent with the results presented in the current study as differences in plasma AA concentrations were not observed between 2 and 4 h in grazing horses.

However, the peak concentrations for individual AAs ranged between 41 and 393 μM for methionine and valine, respectively. In previous research conducted by Graham-Thiers and Bowen (2011), methionine also had the lowest values of 34 and 43 μM in hay and hay plus grain diets, respectively. Trottier et al. (2002) evaluated AA levels in horses who had completed endurance exercise; they found valine levels were lower at 200 and 234 μM for hay and hay grain diets, respectively, and that valine required 48 h to rebound to pre-exercise levels (Trottier et al., 2002). The lower valine levels could be a result of decreased valine in response to exercise. However, DePew et al. (1994) found similar results to the current study with the highest peak values observed in valine, slightly under 400 μM. Their studies had similar peak AA concentrations observed across other AAs; however, DePew et al. (1994) had slightly higher values. This discrepancy could be a result of different feedstuff considering the current study evaluated horses grazing pasture while DePew et al. (1994) observed horses consuming a pellet and hay ration. As a result,

differences may be observed due to alterations in feed composition, intake, or digestibility.

These results help confirm that diet composition and exercise play a crucial role in influencing factors that can impact the availability and absorption of AAs. Key factors include the AA composition, which is influenced by the protein content and relative proportions of AA, as well as digestibility and intake, which are impacted by ADF and NDF, respectively.

Previous research has demonstrated more digestible and available sources of AAs tend to be found in commercial grain mixes compared to hay (Graham-Thiers and Bowen, 2011). Graham-Thiers and Bowen (2011) found that horses consuming grain observed early increases in plasma AA concentrations and an improved N balance compared to horses receiving hay only diets. These results suggested horses consuming both hay and a commercial-grain mix had more available AAs due to the AA composition of the feedstuff and the increased foregut digestibility.

Differences among compositions have also been evaluated in different types of hay to determine the impact forage species had on digestibility of hay protein (Gibbs et al., 1988). Gibbs et al. (1988) evaluated the digestibility of hay protein in ponies consuming coastal bermudagrass in comparison to both a low- and high-protein alfalfa. They found higher N retention in ponies consuming the high-protein alfalfa hay and increased total tract and prececal digestibility. While the large intestine plays the largest role in the fermentation of hay protein, Gibbs et al. (1988) demonstrated that improving the quality of the hay by increasing CP and decreasing fiber values was capable of increasing the role of the small intestine in protein digestibility. These results suggest

forage differences related to CP, NDF, and ADF can play a role in the foregut availability of AA.

The results from the current study support these relationships, as a positive correlation was observed between total plasma AAs and total forage AAs; however, a significant correlation was not observed between total plasma AAs and forage CP. These results suggest total forage AAs are a better predictor of protein that can be digested and AAs that can be absorbed in comparison to CP content which is the current standard. However, while total AAs concentrations are important when evaluating protein, the relationship between total plasma AA and total forage AA was not strong, indicating other factors contribute to these processes.

Additionally, a negative correlation was observed between fiber values, including ADF and NDF, when compared to CP. These results suggest fiber and protein compete for space or resources within the plant. A similar relationship has been observed in studies evaluating the maturity of forages; as forage matures, fiber content increases and CP decreases (Balde et al., 1993; Elizalde et al., 1999). However, in research evaluating alfalfa and orchardgrass fed to cattle, decreased CP content did not impact relative proportions of AAs in the forage (Balde et al., 1993). As a result, in addition to decreasing protein digestibility and availability, fiber content also influences total CP and AA concentrations in the forage but does not alter the AA ratio. Based on characteristics of different forage species or increased maturity of the forage, fiber content can increase with a corresponding decrease in CP and AA concentrations. These factors should be taken into consideration when choosing the proper forage for a horse.

These relationships connect to the current study as lower CP and AA concentrations and higher fiber values were found in teff compared to alfalfa and CSG; teff had approximately 8% less CP, and 7 to 21% more NDF and ADF compared to CSG and alfalfa. Despite these differences, all of the CP values exceeded the NRC 12% DM recommendation for adult horses at maintenance (NRC, 2007) and minimal differences were observed in plasma AA concentrations of grazing horses. However, decreased intake and digestibility corresponding to the higher NDF and ADF levels could influence the horse's ability to meet AA requirements. Additionally, the higher fiber content of warm-season forages could compete with the ability of the forage to acquire protein. Furthermore, relative proportions of plasma AAs were evaluated in relation to total protein. This assessment allows the ratio of AAs to be compared in the horse to determine if a single AA may be limiting. In the current study, differences were observed in relative proportions of threonine in teff when compared to alfalfa. Considering threonine is currently considered one of the most limiting AAs in the horse, insufficiencies of this AA could result in decreased protein synthesis.

However, with the exception of differences observed in threonine concentrations, the relative proportions and total values of essential AA were similar across all forage species in the current study and in comparison to other feedstuffs reported in previous studies (Woodward et al., 2011; Graham-Thiers and Bowen, 2011; DePew et al., 1994). When evaluating lysine and threonine in the current study, forage values ranged from 0.72 to 1.25 % DM and 0.51 and 0.92% DM, respectively. With the exception of teff, these values exceed those fed to horses receiving a pelleted ration (DePew et al., 1994) at 0.78 and 0.58 % DM as well as alfalfa hay (Woodward et al., 2011) at 0.73 and 0.62 %

DM, for lysine and threonine, respectively. Additionally, AA compositions from other feedstuff, including bermudagrass hay (DePew et al., 1994), timothy hay (Woodward et al., 2011), and a commercial grain mix and cool-season grass hay (Graham-Thiers and Bowen, 2011) contained less than 0.51 and 0.40% DM for lysine and threonine, respectively. These results suggest pasture, in the form of alfalfa, CSG, or teff, can supply comparable or greater AA concentrations in comparison to other feedstuffs currently fed to horses.

While relative proportions of AAs are important, lysine is currently believed to be the most limiting AA and the only AA for which requirements have been established by the NRC (2007) for the horse. The minimum requirement of a 500 kg horse at maintenance is 23.2 grams per day up to 84.8 grams per day which is the maximum requirement for a lactating mare. Based on the findings from the current study, if a horse consumed 1.5% BW of fresh pasture on a DM basis, they would consume 88, 94, or 54 g lysine for alfalfa, CSG, or teff, respectively, suggesting these forages can meet the requirements of most horses, with the exception of lactating mares grazing teff at 1.5% BW.

In the future, more research is needed to evaluate how intake and digestibility of horses consuming different forage may influence nutrient consumption and availability. Additionally, an extended consumption of the forages, specifically to evaluate tissue utilization of AAs, would be useful in determining the long-term impacts of pasture forage consumption on the horse.

CONCLUSION

In conclusion, while significant differences were observed in the AA composition of different forage species, significant differences in plasma concentrations were only observed for threonine after 4 h of grazing. These results suggest all AA were absorbed or available in sufficient amounts regardless of differences in the forage species. The lack of differences in *de novo* protein synthesis suggest that while DE, CP, fiber content and AA amounts differed across forages, all forage species were able to support similar protein synthesis initiation in cultured equine skeletal muscle cells. These results suggest that differences among forage AA concentration and fiber content did not elicit different physiological responses in equine skeletal muscle.

Table 2.1. Nutrient composition on a dry matter (DM) basis for alfalfa, cool-season grass (CSG), and teff grazed by mature mares in July in St. Paul, MN during 2016.

Nutrient[1]	Alfalfa	CSG	Teff	SE
DE (Mcal kg^{-1})	2.37[a†]	2.22[a]	2.01[b]	0.02
NSC (%)	9.8[ab]	11.3[a]	8.4[b]	0.6
CP (%)	23.3[a]	24.1[a]	15.4[b]	0.7
NDF (%)	43.2[c]	53.4[b]	65.0[a]	1.1
ADF (%)	33.1 [ab]	30.9[b]	37.0[a]	0.5
Amino Acid (%)				
Arginine	0.89[ab]	1.07[a]	0.57[b]	0.04
Cystine	0.22[ab]	0.25[a]	0.14[b]	0.01
Histidine	0.43[a]	0.43[a]	0.23[b]	0.02
Isoleucine	0.95[a]	1.01[a]	0.58[b]	0.04
Leucine	1.57[ab]	1.75[a]	1.02[b]	0.07
Lysine	1.17[a]	1.25[a]	0.72[b]	0.05
Methionine	0.33[ab]	0.41[a]	0.24[b]	0.02
Phenylalanine	1.02[ab]	1.18[a]	0.65[b]	0.05
Threonine	0.81[a]	0.92[a]	0.51[b]	0.03
Tryptophan	0.28	0.30	0.15	0.02
Tyrosine	0.57[ab]	0.61[a]	0.33[b]	0.03
Valine	1.16[a]	1.28[a]	0.74[b]	0.05

[1]Digestible energy (DE), nonstructural carbohydrates (NSC), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and amino acid concentrations.
[a-b]Within a row, means without a common letter superscript differ based on a Tukey test ($P \leq 0.05$); means without a superscript were not different ($P > 0.0$).

Table 2.2 Amino acid concentrations (μM) in plasma of horses consuming alfalfa, cool-season grass (CSG), and teff pastures prior to turn-out (0 h), and 2 and 4 h post-turnout in July 2016 in St. Paul, MN.

	Hour				Hour				Hour		
	0	**2**	**4**		**0**	**2**	**4**		**0**	**2**	**4**
	Lysine				**Histidine**				**Arginine**		
Alfalfa	76.0	244.6	229.4	**Alfalfa**	63.8	96.8	98.1^{x†}	**Alfalfa**	58.2	159.4	154.3
CSG	75.4	265.4	250.9	**CSG**	64.9	100.6	98.0^{x}	**CSG**	57.8	163.0	158.0
Teff	79.1	223.1	197.7	**Teff**	62.9	95.2	90.5^{y}	**Teff**	61.6	150.4	138.5
SE	1.6	15.8	15.9	**SE**	1.0	1.8	1.8	**SE**	0.8	10.7	10.8
	Leucine				**Isoleucine**				**Valine**		
Alfalfa	90.9	211.1	231.3	**Alfalfa**	49.0	122.2	134.7	**Alfalfa**	174.6	312.6	357.0
CSG	89.9	234.8	258.4	**CSG**	50.6	124.2	136.2	**CSG**	175.0	340.5	396.8
Teff	99.6	193.7	205.5	**Teff**	53.3	103.8	103.8	**Teff**	181.9	298.0	327.4
SE	2.3	20.9	21.1	**SE**	0.7	9.8	9.8	**SE**	2.4	22.5	22.5
	Threonine				**Methionine**				**Cysteine**		
Alfalfa	79.8	192.9	226.8^{ab*}	**Alfalfa**	22.0	41.3	41.4^{y†}	**Alfalfa**	46.4	44.6	39.8
CSG	83.7	215.0	251.9^{a}	**CSG**	22.0	49.2	54.8^{x}	**CSG**	45.9	46.3	43.6
Teff	84.4	182.4	196.2^{b}	**Teff**	22.4	42.0	41.3^{y}	**Teff**	45.5	48.6	43.9
SE	2.5	9.0	9.0	**SE**	0.2	2.7	2.7	**SE**	0.3	1.5	1.5
	Phenylalanine				**Tryptophan**						
Alfalfa	46.6	80.4	86.9	**Alfalfa**	45.5	74.5	80.4				
CSG	46.6	90.3	104.6	**CSG**	45.1	81.8	89.6				
Teff	47.3	76.1	80.7	**Teff**	44.6	72.8	75.0				
SE	1.2	5.2	5.2	**SE**	1.4	5.4	5.4				

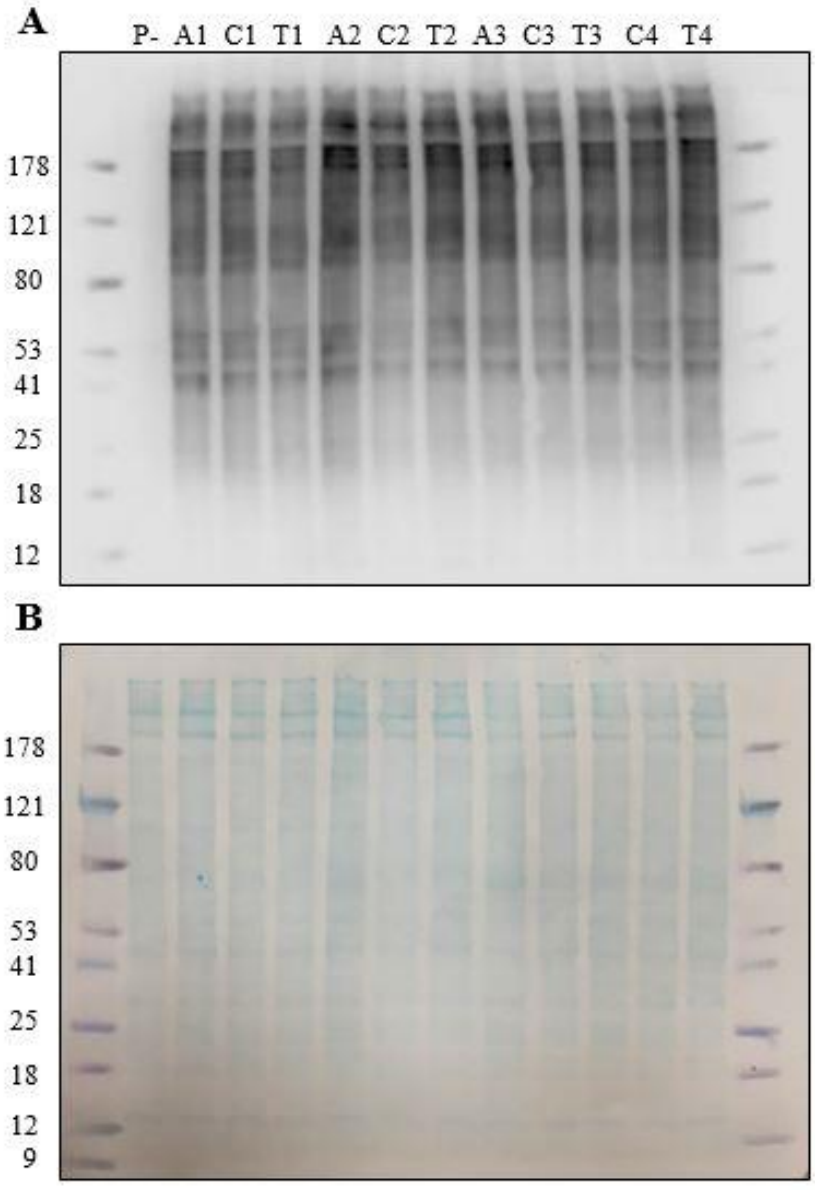
$^{a-b}$Within a column, means without a common letter superscript differ based on a Tukey test ($P \leq 0.05$).
$^{x-y}$Within a column, means without a common letter superscript differ based on a Tukey test ($P \leq 0.10$); means without a superscript were not different ($P > 0.10$).

Table 2.3. Amino acid concentrations relative to total protein[1] in plasma of horses consuming alfalfa, cool-season grass (CSG), and teff pastures prior to turn-out (0 h), and 2 and 4 h post-turnout in July 2016 in St. Paul, MN.

	Hour				Hour				Hour		
	0	**2**	**4**		**0**	**2**	**4**		**0**	**2**	**4**
	Lysine				**Histidine**				**Arginine**		
Alfalfa	4.2	7.6	6.9	**Alfalfa**	3.6	3.0	3.0	**Alfalfa**	3.3	5.0	4.7
CSG	4.2	7.4	6.6	**CSG**	3.7	2.9	2.6	**CSG**	3.2	4.6	4.2
Teff	4.3	6.8	6.1	**Teff**	3.5	3.0	2.9	**Teff**	3.4	4.6	4.3
SE	0.1	0.3	0.3	**SE**	0.1	0.1	0.1	**SE**	0.1	0.2	0.2
	Leucine				**Isoleucine**				**Valine**		
Alfalfa	5.2	6.6	7.0	**Alfalfa**	2.8	3.8	4.1	**Alfalfa**	9.9	9.7	10.9
CSG	5.1	6.6	6.9	**CSG**	2.9	3.5	3.6	**CSG**	10.0	9.5	10.5
Teff	5.5	6.0	6.5	**Teff**	2.9	3.2	3.3	**Teff**	10.1	9.4	10.5
SE	0.2	0.4	0.4	**SE**	0.1	0.2	0.2	**SE**	0.4	0.4	0.4
	Threonine				**Methionine**				**Cysteine**		
Alfalfa	4.4	6.0	6.9[a]	**Alfalfa**	1.2	1.3	1.3	**Alfalfa**	2.6	1.4	1.2
CSG	4.5	6.0	6.7[ab]	**CSG**	1.2	1.4	1.5	**CSG**	2.6	1.3	1.2
Teff	4.7	5.6	6.1[b]	**Teff**	1.2	1.3	1.3	**Teff**	2.5	1.5	1.4
SE	0.2	0.1	0.1	**SE**	0.04	0.05	0.05	**SE**	0.1	0.1	0.1
	Phenylalanine				**Tryptophan**						
Alfalfa	2.6	2.5	2.6	**Alfalfa**	2.6	2.3	2.5				
CSG	2.6	2.5	2.8	**CSG**	2.5	2.3	2.4				
Teff	2.6	2.4	2.5	**Teff**	2.5	2.2	2.3				
SE	0.1	0.1	0.1	**SE**	0.1	0.1	0.1				

[1]Total protein is the sum of lysine, histidine, arginine, leucine, ileucine, valine, threonine, methionine, cysteine, phenylalanine, tryptophan, tyrosine, glutamic acid, glutamine, aspartic acid, asparagine, alanine, serine, glycine, and proline.
[a,b]Within a column, means without a common letter superscript differ based on a Tukey test ($P \leq 0.05$); means without a superscript were not different ($P > 0.05$).

Figure 2.1. (A) Western blot analysis for puromycin incorporation into nascent proteins in equine myotube cell cultures treated with 3% sera pooled from horses (n = 6) grazing alfalfa (A), cool-season grass (C) or teff (T) and analyzed in triplicate or quadruplicate. Treatment was applied for 3 h with puromycin applied for the final 1 h. The specificity of the anti-puromycin blot was demonstrated by a sample that was not incubated with puromycin (P-). Band densities of the entire lane for each of the lanes per treatment were quantified and averaged to compare alfalfa, cool-season grass, and teff cultures; alfalfa had an average lane band density of 1,028,000 ± 45,468, in comparison to cool-season grass at 921,750 ± 39,376 and teff at 1,003,250 ± 39,376. No differences were observed between equine myotube cultures treated with horse plasma from horses grazing the different forage species ($P \geq 0.2$). (B) The reversible protein stain was used to verify equal loading.



LITERATURE CITED

Balde, A.T., J.H. Vendersall, R.A. Erdman, J.B. Reeves III, and B.P. Glenn. 1993. Effect of stage of maturity of alfalfa and orchardgrass on in situ dry matter and crude protein degradability and amino acid composition. Anim. Feed Sci. Technol. 44:29-43.

Bott, R.C., E.A. Greene, N.L. Trottier, C.A. Williams. M.L. Westendorf, A.M. Swinker, S.L. Mastellar, and K.L. Martinson. 2016. Environmental implications of nitrogen output on horse operations: a review. J. Equine Vet. Sci. 45:98 – 106.

DeBoer, M.L., K.M. Martinson, M.S. Pampusch, A.M. Hansen, S.M. Wells, C. Ward, and M. Hathway. 2018. Cultured equine satellite cells as a model system to assess leucine stimulated protein synthesis in horse muscle. J. Anim. Sci. 96(1):143-153.

DePew, C.L., D.L. Thompson, Jr., J.M. Fernandez, L.S. Sticker, and D.W. Burleigh. 1994. Changes in concentrations of hormones, metabolites, and amino acids in plasma of adult horses relative to overnight feed deprivation followed by a pellet-hay meal fed at noon. J. Anim. Sci. 72: 1530-1539.

Elizalde, J.C., N.R. Merchen, and D.B. Faulkner. 1999. Fractionation of fiber and crude protein in fresh forages during the spring growth. J. Anim. Sci. 77:476-484.

Gibbs, P.G., G.D. Potter, G.T. Schelling, J.L. Krieder, and C.L. Boyd. 1988. Digestion of hay protein in different segments of the equine digestive tract. J. Anim. Sci. 66:400-406.

Graham-Thiers, P.M., and L.K. Bowen. 2011. Effect of protein source on nitrogen balance and plasma amino acids in exercising horses. J. Anim. Sci. 89:729-735.

Hall, M.B., W.B. Hoover, J.P. Jennings, and T.K. Miller. 1991. A method for partitioning neutral detergent soluble carbohydrates. J. Sci. Food Agric. 79:2079-2086.

Henneke, D.R., G.D. Potter, J.L. Kreider, and B.F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15(4):371–372.

Johnson, R.J., and J.W. Hart. 1974. Influence of feeding and fasting on plasma free amino acids in the equine. J. Anim. Sci. 38(4): 790-794.

Kalu, B.A, and G.W. Fick. 1983. Morphological stage of development as a predictor of alfalfa herbage quality. Crop Sci. 23:1167-1172.

Littel, R.C., P.R. Henry, and C.B. Ammerman. 1998. Statistical anaylsis of repeated measures data using SAS procedures. J. Anim. Sci. 76: 1216-1231.

Martinson, K., M. Hathaway, J.H. Wilson, B. Gilkerson, P.R. Peterson, and R.D. Vecchio. 2006. University of Minnesota Horse Owner Survey: Building an equine extension program. J. Extension. 44(6):6RIB4.

Moore, K.J., L.E. Moser, K.P. Vogel, S.S. Waller, and B.E. Johnson. 1991. Describing and quantifying growth stages of perennial forage grasses. Agron. J. 83:1073-1077.

Nakano, K., and H. Hara. 1979. Measurement of the protein-synthetic activity in vivo of various tissues in rates by using [3H]Puromycin. Biochem. J. 184: 663-668.

Nathans, D. 1964. Puromycin inhibition of protein synthesis: incorporation of puromycin into peptide chains. Proc. Natl. Acad. Sci. U.S.A. 51(4): 585 – 592.

NRC. 2007. Nutrient requirements of horses. 6th ed. The National Academies Press, Washington, D.C.

Pagan, J.D. Measuring the digestible energy content of horse feeds. In Advanced Equine Nutrition. Inited Kingdom: Nottingham University Press; 1998. p. 71-76.

Reid, R.L., G.A. Jung, and W.V. Thayne. 1988. Relationships between nutritive quality and fiber components of cool season and warm season forages: a retrospective study. J. Anim. Sci. 66:1275-1291.

Russell, M.A., A.V. Rodiek, and L.M. Lawrence. 1986. Effect of meal schedules and fasting on selected plasma free amino acids in horses. J. Anim. Sci. 63: 1428-1431.

Schmidt, E.K., G. Clavarino, M. Ceppi, and P. Pierre. 2009. SUnSET, a nonradioactive method to monitor protein synthesis. Nat. Methods. 6: 275 – 277.

Straková, E., P. Suchý, I. Král, D. Zapltal, and I. Herzig. 2013. The content of amino acids in pasture vegetation and their apparent digestibility in 2-year-old horses. J. Equine Vet. Sci. 33:984-988.

Trottier, N.L., B.D. Nielsen, K.J. Lang, P.K. Ku, and H.C. Schott. 2002. Equine endurance exercise alters serum branched-chain amino acid and alanine concentrations. Equine Vet. J. 34: 168-172.

Wilson, J.R. 1994. Cell wall characteristics in relation to forage digestion by ruminants. J. Agr. Sci. 122:173-182.

Woodward, A.D., B.D. Nielsen, J. Liesman, T. Lavin, and N.L. Trottier. 2011. Protein quality and utilization of timothy, oat-supplemented timothy, and alfalfa at differing harvest maturities in exercised Arabian horses. J. Anim. Sci. 89:4081-4092.

Chapter 3

Glucose and insulin response of horses grazing alfalfa, cool-season perennial grass, and teff during the summer and fall

M.L. DeBoer[a], M.R. Hathaway[a], K.J. Kuhle[b], P.S.D. Weber[c], A.S. Reiter[a], C.C. Sheaffer[d], M.S. Wells[d], K.L. Martinson[a]*

[a]Department of Animal Science, University of Minnesota, St. Paul, MN

[b]Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN

[c]Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, MI

[d]Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN

SUMMARY

Elevated nonstructural carbohydrate (NSC) values in pasture forages can cause adverse health effects in some horses (*Equus caballus* L.). The objectives of this study were to determine the impact of different forage species on blood glucose and insulin concentrations of horses throughout the grazing season. Research was conducted in July (summer) and September (fall) in St. Paul, MN. Alfalfa (*Medicago sativa L.*), mixed perennial cool-season grasses (CSG), and teff (*Eragrostis tef* [Zucc.] Trotter) pastures were grazed by six horses (24 ± 2 yr) who were randomly assigned to one of three forage types in a replicated Latin-square design. Jugular catheters were inserted 1 h prior to the start of grazing and horses had access to pasture each day from 0800 h to 1600 h. Jugular

venous blood samples were collected from each horse prior to being turned out (0 h) and then at 2 hour intervals following turnout. Plasma and serum samples were collected and analyzed for glucose and insulin, respectively. Corresponding forage samples were taken by hand harvest. Seasons were analyzed separately and data were analyzed using the MIXED procedure in SAS with $P \leq 0.05$. Teff generally had lower ($P \leq 0.05$) equine digestible energy (DE), crude protein (CP) and NSC compared to the other forages. Differences in peak insulin were observed between horses grazing CSG and teff during the fall grazing ($P \leq 0.05$). These results suggest grazing teff could lower the glucose and insulin response of some horses.

INTRODUCTION

Obesity, insulin resistance (IR), laminitis, and Equine Metabolic Syndrome (EMS) are growing concerns in the horse industry. Experts estimate that 19 to 40% of the horse population is obese (Wyse et al., 2008; Stephenson et al., 2011; Thatcher et al., 2012; Giles et al., 2009) and 22 to 29% are hyperinsulinemic (Muno et al., 2009; Morgan et al., 2014). Aged horses may be at a higher risk for these conditions due to decreased exercise, development of metabolic diseases (McGowan et al., 2013), and larger insulinemic responses which have the capability to lead to hyperinsulinemia or insulin dysregulation (Vick et al., 2007; Jacob et al., 2017). Fortunately, management modifications have helped improve the care of horses diagnosed with these metabolic dysfunctions including restricting access to pasture and feeding a high-fiber, low NSC diet (Frank. 2011).

Regardless of their horse's disease status, many owners desire pasture access for their horses. However, pasture access may have a detrimental impact on a diseased

horse's health due to the lower fiber and higher NSC values of many pasture forages compared to the same forages dried in hay (Allen et al., 2013). Across much of the United States, CSG are the primary forage in horse pastures. However, CSG tend to have greater amounts of NSC compared to warm-season grasses and legumes (Catalano et al., 2015; DeBoer et al., 2017; Grev et al., 2017). Although some research is available on the glucose and insulin response of horses grazing a single pasture species (McIntosh et al., 2007; Staniar et al., 2007; Richards and Kempton, 2016), little information is available on the effect of horses grazing different pasture species and impacts on the glucose and insulin response. While differences in nutritive values among forage species are known, it is unclear if these differences will elicit a unique glucose and insulin response in horses. Therefore, this study investigated the glucose and insulin response of horses grazing alfalfa, CSG, and teff throughout the grazing season. The hypothesis was horses consuming CSG would have a higher glucose and insulin responses compared to horses grazing teff with intermediary results observed in horses grazing alfalfa.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Horse Management

Six mares (24 ± 2 yr) were body condition scored (Henneke et al., 1983) and challenged with an oral sugar test (OST; Table 3.1) prior to the start of the study (Schuver et al., 2014). One horse (Horse 6) died unexpectedly following the summer grazing and was replaced with another horse (Horse 7) for the fall grazing period; the horse's death was not related to the present research.

Horses had *ad libitum* access to water throughout the study and when not grazing, horses were housed in a dry lot and fed a mixed hay containing equal parts alfalfa, CSG and teff at approximately 2.5% bodyweight (BW) split evenly between two daily feedings. Between the two grazing periods, horses grazed CSG or alfalfa pastures during the day and were housed in a dry lot overnight with *ad libitum* access to CSG hay. Horses were also fed a ration balancer (Enrich Plus Ration Balancing Horse Feed, Purina, St. Louis, MO) at 0.1% BW at 1700 h each day to ensure all vitamin and mineral requirements were met for adult horses at maintenance (NRC, 2007).

Experimental Design and Diets

Horses were randomly assigned to three forage types over three days in a 3 x 3 Latin-square design. Forages consisted of alfalfa, CSG (mixture of orchardgrass (*Dactylis glomerata* L.) and Kentucky bluegrass (*Poa pratensis* L.)), and teff. Alfalfa stands were established on May 2014 in a 0.07 ha pasture and CSG pastures were established on August 2009 in a 0.17 ha pasture. A 0.17 ha teff pasture was established on June 2016 and seeded at a rate of 13.5 kg ha^{-1}. The soil was a Waukegan silt loam (fine-silty over skeletal, mixed, superactive, mesic Typic Hapludoll) with a soil pH of 6.6, 18 ppm P, and 85 ppm K, 13 ppm NO_3-N; no fertilization was needed based on soil test results.

Average forage maturity was assessed prior to grazing. Alfalfa maturity was assessed using the mean stage count method (Kalu and Fick, 1983), while maturity for CSG and teff was determined using a scale developed by Moore et al. (1991). Alfalfa was grazed at the early bud stage in the summer and the early flower stage in the fall with the average maturity of 3 and 5 in the summer and fall, respectively. The CSG pasture was grazed at a late vegetative stage across seasons. Teff was grazed in the stem elongation

and inflorescence emergence phase for summer and fall, respectively. The average grazing height for the forages prior to turnout was 58, 42, and 55 cm for alfalfa, CSG, and teff, respectively.

All pastures were mowed to 8 cm three weeks prior to the start of each grazing period to allow for an equal regrowth period. Each pasture was then divided into three equal subplots to allow each horse group (n = 2) access to fresh, un-grazed pasture during the period. Each pasture subplot had sufficient forage available that allowed horses to graze *ad libitum* throughout the 8 hour grazing period. During the summer and fall, forages were grazed on July 19, 21, and 23 and September 12, 14, and 16, respectively, from 0800 to 1600 h. Prior to the start of each grazing event, horses received a 24 h hay washout consisting of equal amounts of the three forage species followed by a 12 h fast. Upon completion of blood collection, horses repeated the hay washout and fasting period before switching treatments. Upon completion of grazing each day, manure was removed from the pastures and forages were mowed to 8 cm and allowed to re-grow.

Sampling and Analysis

Indwelling catheters were inserted approximately 1 h prior to the start of blood collection using a local anesthetic (2% lidocaine, Lidocaine 20 mg ml^{-1}, VetOne, MWI Animal Health. Boise, ID) blockade. Blood samples were then taken prior to turnout at 08:00 (0 h) and 2, 4, 6 and 8 h post-turnout, at 10:00, 12:00, 14:00, and 16:00 h, respectively. Serum samples were collected in 9-mL serum-separator tubes (8881302015; Covidien, Minneapolis, MN) and left at room temperature for 45 minutes following collection. Plasma samples were collected in 10-mL tubes with an EDTA-additive (8881311743; Covidien, Minneapolis, MN) and put on ice immediately after collection.

Following blood collection, catheter lines were flushed with 10 mL of heparinized saline (1,000 units of heparin 200 mL^{-1} of 0.9% saline). Serum and plasma samples were separated by centrifugation at 1,200 x g at 4°C for 20 minutes, supernatants were collected, aliquoted and stored at -80°C for later analysis.

Glucose concentrations were determined in duplicate by a membrane based glucose oxidase method (YSI 2300 STAT Plus™ Glucose & Lactate Analyzer; YSI Incorporated Life Sciences, Yellow Springs, OH) using plasma samples. Insulin concentrations were determined in duplicate serum samples using the EMD Millipore Porcine Insulin Specific RIA Kit (PI-12K; EMD Millipore Porcine Insulin Specific RIA Kit; Billerica, MA, USA) previously validated for use in equine serum (Warnken et al., 2016). Intra- and inter-assay coefficients of variability (CVs) were calculated using pooled equine serum samples containing low and high concentrations of insulin. Intra- and inter-assay CVs for the low serum sample were 6.1% and 5.8%, respectively and for the high serum sample the CVs were 7.4% and 8.4%.

Duplicate, representative forage samples were taken from each pasture at 08:00, 10:00, 12:00, 14:00, and 16:00 to correspond to blood samples. Samples were clipped at 8 cm and dried for 24 h at 60º C. After drying, samples were ground through a 6-mm screen in a Wiley mill (Thomas Scientific, Swedesboro, NJ) followed by a 1-mm screen in a Cyclotec (Foss, Eden Prairie, MN). Samples were mixed thoroughly and subsamples were analyzed for forage nutritive value by a commercial forage testing laboratory (Equi-Analytical, Ithaca, NY). Crude protein was calculated as the percentage of N multiplied by 6.25, a method determined by the Association of Official Agricultural Chemists (method 990.03). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were

measured using filter bag techniques of ANKOM Technology. Starch concentrations were determined using a glucoamylase enzyme and by measuring dextrose in an automated biochemical analyzer (YSI 2700 select biochemistry analyzer, YSI Incorporated, Yellow Springs, OH). Ethanol soluble carbohydrates (ESC) and water soluble carbohydrates (WSC) were measured using techniques described by Hall et al. (1991). Non-structural carbohydrates were mathematically estimated by adding WSC plus starch. Equine DE was calculated using an equation developed by Pagan (1998).

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) as a replicated 3 x 3 Latin-square design. Day and pen were blocking factors (i.e. rows and columns) in the Latin-square design. Each replicate was the experimental unit for forage. Variables analyzed included equine DE, starch, WSC, ESC, NSC, ADF, NDF, and CP. Data was analyzed using a repeated measures design according to methods used by Littel et al. (1998). The model included treatment, day, pen, hour, and treatment × hour.

Individual horses within a forage treatment were the experimental unit for glucose and insulin concentrations. Variables analyzed included baseline, average, and peak values for both glucose and insulin in the horses. Blood samples taken at hour 0 were included in the model as a baseline covariate for peak and average glucose and insulin values. The average blood values were analyzed using a repeated measures design (Littel et al., 1998). The model included the baseline covariate as well as treatment, day, pen, hour, and treatment × hour. A square root transformation was used for insulin values in order to meet ANOVA assumptions; data was back transformed for presentation. Means

are averaged over the sampling days within the season with the least square means of the MIXED procedure (± SE), and mean separations determined using Tukey's HSD test ($P \leq 0.05$). Based on the influencing statistics, Horse 1 was considered an outlier and was not included in the analysis. Glucose and insulin area under the curve (AUC) were not reported as a result of differences in grazing time across seasons. Additionally, blood values did not return to baseline within the 8 h grazing period. Statistical significance was set at $P \leq 0.05$ with trends identified at $P \leq 0.10$.

RESULTS

Environment

Environmental conditions are reported in Table 3.2. The environmental conditions observed were similar to historical averages for St. Paul, MN.

Forage Nutritive Values

Differences in forage nutritive values were observed between the different species for both seasons (Table 3.3). In the summer and fall, alfalfa had the greatest equine DE ($P \leq 0.01$) with the lowest equine DE values observed in teff. The only difference observed in NSC was during the summer when teff was lower in NSC compared to CSG ($P \leq 0.01$). In the summer and fall, NDF was highest in teff and lowest in alfalfa ($P \leq 0.01$) while the ADF was highest in teff and lowest in CSG ($P \leq 0.01$). In both seasons, CP was highest in alfalfa and lowest in teff.

Changes in NSC were also evaluated throughout the 8 h grazing period within each season (data not shown). In the summer, NSC values ranged from 9.2 to 10.7% in alfalfa, 9.8 to 12.6% in CSG, and 7.3 to 9.5% in teff. However, no differences were observed between the different time points. In the fall, NSC values ranged from 7.4 to

11.0% in alfalfa, 9.3 to 11.1% in CSG, and 7.5 to 8.2% in teff. While no differences were observed in CSG and teff across the time points, differences in alfalfa NSC were found. Higher NSC content in alfalfa was observed at h 8 compared to 0, 2 and 4 h.

Glycemic and Insulinemic Response

Baseline glucose values ranged from 88 to 89 mg dL^{-1} in the summer and 86 to 87 mg dL^{-1} in the fall with no differences observed ($P \geq 0.05$; Table 3.4). Additionally, neither average nor peak glucose was different for horses grazing alfalfa, CSG of teff, regardless of season (Table 3.4). Baseline insulin values ranged from 7.4 to 8.8 μIU mL^{-1}, with no differences observed ($P \geq 0.05$; Table 3.4). No differences were observed in average insulin values or peak insulin values in the summer (Table 4). However, horses grazing teff had lower peak insulin values in the fall when compared to CSG (Table 3.4, $P \leq 0.05$). When evaluating differences in the blood characteristics between horses grazing the different forage species at each time point post-turnout, no differences were observed (data not shown).

DISCUSSION

Forage Nutritive Values

Nutritive values of feedstuffs are crucial to properly balancing horse rations, especially for horses diagnosed with metabolic diseases. Frank (2009) suggested a total diet ≤12% NSC for horses diagnosed with EMS and Borgia et al. (2009) recommended hay containing ≤10% NSC for horses affected by polysaccharide storage myopathy. Furthermore, Staniar et al. (2007) observed a relationship between sugar and starch and the glycemic and insulinemic responses of horses, Rodiek and Stull (Rodiek and Stull, 2007) observed a relationship between NSC and the glycemic index of horses and

Gordon et al. (Gordon et al., 2007) determined a low NSC meal would lead to a lower glucose and insulin response. However, age could alter these recommendations as decreased insulin sensitivity has been observed in aged horses (Jacob et al., 2017).

In the current study, CSG contained higher NSC levels in comparison to teff during the summer. This was anticipated since CSG stores excess carbohydrates in the form of fructans which can be translocated to the stem (Chatterton et al., 1989). In comparison, legumes and warm-season grasses have a self-limiting carbohydrate storage mechanism. As a result, lower NSC values are often observed in legumes and warm-season species when compared to CSG species (Chatterton et al., 1989). While minimal research has compared the NSC content of these different forage species, DeBoer et al. (2017) found teff pastures averaged ≤9% NSC and Staniar et al. (2010) observed NSC contents ranging from 5 to 8% in teff hay. Additionally, Rodiek and Jones (2012) determined that alfalfa and teff hay had NSC levels ≤12% while oat and wheat hay had NSC values ≥30%. In comparison, cool-season perennial grasses have reported NSC values ranging from 6 to 17% (Allen et al., 2013) while cool-season annual grasses had NSC values ranging from 10 to 22% (Grev et al., 2017). Considering feedstuffs with ≤12% NSC have been suggested as a low NSC feed for horses (Frank, 2011; Borgia et al., 2009) based on the current study, both teff and alfalfa would be considered low NSC feeds. However, variables including maturity, environmental conditions and management decisions could have an impact on NSC concentrations and should be considered in specific cases and future research.

Diurnal variations can also impact the NSC content of forages with NSC values increasing throughout the day and decreasing overnight (Morin et al., 2011; Morin et al.,

2012) While the current study evaluated the NSC content during a typical daytime grazing period, from 08:00 to 16:00 h, changes in NSC were only observed in alfalfa in the fall. Future research should evaluate impacts of these forages on blood metabolites over a 24 h period. Another variable that can influence NSC is the preservation of plant materials following cutting. In the current study, plants were subject to oven-drying at 60°C for 24 h prior to nutritive analysis. Recent studies prefer freeze-drying or microwave pretreatment to inactivate hydrolytic enzymes which can alter the carbohydrate values in a plant (Pelletier et al., 2010). As a result, NSC values reported in the current study may be lower than the actual NSC concentrations present in the fresh pasture.

Recent studies have determined that NSC content alone is not a good predictor of glucose and insulin response of horses consuming feedstuffs. In the current study, NSC did not vary across forage species in the fall ($P > 0.05$); however, variations in peak insulin were observed between horses consuming teff and CSG pastures during that season. Although consistent re-growth periods were given, plant species were at different maturities and heights during grazing. Siciliano et al. (2017) found a greater insulinemic response in horses grazing tall versus shorter sward heights. As a result, variations in sward heights due to different forage growth habits could have contributed to different responses observed in the current study.

In addition to considering the role of growth patterns and sward height in glucose and insulin response, Richards and Kempton (2016) found that digestibility of NSC played an important role in the glycemic and insulinemic response, and NSC alone was incapable of predicting the response. Teff consistently had higher NDF and ADF values

and horses grazing teff had a lower insulinemic response in the fall. Fiber concentrations play an important role in digestibility of a feedstuff and likely influenced the insulin response of grazing horses (Richards and Kempton, 2016).

While the nutritive components of a feed are important, the long term effects of the feedstuff on the physiological response of the horse should also be considered. Since obesity is one of the major contributing factors for horses diagnosed with EMS and insulin resistance (Allen et al., 2013; Carter et al., 2009), weight management is important. The equine DE content of a feedstuff is commonly associated with bodyweight maintenance, loss, or gain. The equine DE of teff was 0.2 to 0.5 Mcal kg^{-1} lower compared to CSG and alfalfa, respectively. Based on the DE requirements for an adult horse at maintenance (NRC, 2007), a horse consuming a total forage diet at 1.5% BW would lose one body condition score (Henneke et al., 1983) in 2.5 months when consuming teff, or gain 1 BCS in 3 months when consuming alfalfa. Horse consuming CSG would maintain their bodyweight. Future research should explore if teff is able to elicit BW loss in overweight horses over a longer time period.

Glycemic and Insulinemic Response

In the current study, baseline glucose values ranged from 86 to 89 mg dL^{-1}, which is comparable to values (84 to 96 mg dL^{-1}) observed in previous research (Staniar et al., 2007; Stull and Rodiek 1988; Williams et al., 2001). The peak glucose values observed in the current study ranged from 94 to 101 mg dL^{-1}, which is also comparable to past research where values ranged from 99 to 104 mg dL^{-1} (Staniar et al., 2007; Siciliano et al., 2017).

Baseline insulin values of 7 to 9 μIU mL^{-1} were observed in the current study. These values were slightly lower compared to values of 10.8 and 13.4 μIU mL^{-1} observed by Siciliano et al. (2017). Peak insulin values ranged from 32 to 40 μIU mL^{-1} and 39 to 61 μIU mL^{-1} during the summer and fall grazing periods, respectively. Peak insulin values were greater than those observed by Staniar et al. (2007) and Siciliano et al. (2017) who observed values ≤ 43 and 31 μIU mL^{-1}, respectively. However, these differences could be a result of seasonal variations, as peak insulin concentrations were ≤ 39 μIU mL^{-1} during the summer grazing in the current study. Additionally, the previous studies evaluated blood glucose and insulin during continuous grazing while the current study evaluated blood glucose and insulin following a 12 h fast; which could have contributed to these differences.

Average insulin concentrations while grazing ranged from 23 to 26 uIU mL^{-1} and 33 to 41 uIU mL^{-1} during the summer and fall, respectively. McIntosh et al. (2007) observed mean insulin concentrations as low as 10.9 μIU mL^{-1} when evaluating grazing horses across seasons on CSG. The higher insulin response observed in the current study could be a result of differences in horse age (Vick et al., 2007; Jacob et al., 2017), forage nutritive values (Rodiek and Stull, 2007), management decisions including sward height at the time of grazing (Siciliano et al., 2017), use of fertilizer (McGrath, 1992), individual horses, and fasting prior to consumption (Forhead and Dobson, 1997). However, a limitation to this study is the small sample size used to evaluate the response which may explain the lack of differences observed. Future research utilizing a larger sample size is necessary to confirm these results.

Furthermore, aged horses, regardless of body condition, have demonstrated decreased insulin sensitivity which can put these horses at a higher risk of becoming hyperinsulinemic or developing insulin dysregulation (Vick et al., 2007; Jacob et al., 2017). Jacob et al. (2017) found that horse age influenced peak insulin concentrations, baseline insulin, peak insulin and area under the curve insulin with higher values observed in aged compared to adult horses. These results suggest age is an important factor when considering glucose and insulin responses in horses. Future research should include different age groups when evaluating glucose and insulin responses from grazing different forages.

CONCLUSIONS

The results from this study are some of the first to compare the glycemic and insulinemic responses of horses grazing different pasture species across seasons. While no differences were observed in the glucose response of horses grazing different forage species, horses grazing teff had a lower peak insulin response in the fall when compared to horses grazing CSG. While most of the nutritional differences were observed between CSG and teff, the high equine DE concentrations consistently observed in alfalfa suggest it is most suitable for horses with high energy requirements including performance horses or pregnant and lactating mares. The lower insulin value observed in horses grazing teff, combined with the lower equine DE content and nutrient profile, suggests it could be beneficial as a grazing option for horses requiring an attenuated insulinemic response as well as bodyweight loss commonly associated with metabolic issues.

Table 3.1. Age, breed, BCS, and insulin values from an oral sugar test (OST) at 0 and 90 min for horses used in a grazing study in St. Paul, MN immediately prior to study initiation.

Horse	Age, years	Breed	BCS	Oral Sugar Test Insulin, μIU mL^{-1}	
				0 min	90 min
1	25	Appaloosa	8	19.9	110.0
2	28	Arabian	8	14.7	69.6
3	23	American Quarter Horse	5	17.2	45.5
4	23	American Paint Horse	6	9.0	40.1
5	21	American Paint Horse	5	5.6	21.9
6	26	Thoroughbred	6	7.0	20.9
7	23	American Quarter Horse	6	6.9	9.0

Table 3.2. Mean environmental conditions between 0800 and 1600 h in July (summer) and September (fall) in St. Paul, MN during the 2016 grazing season. Weather data obtained from http://www.dnr.state.mn.us/climate/historical/index.html.

	Summer			Fall		
Days[1]	1	2	3	1	2	3
Mean temperature (°C)	29.5	30.9	24.6	24.2	17.9	21.2
Total precipitation (cm)	0.01	0	0.85	0	0	0
Mean solar Radiation (W M^{-2})	58.6	59.7	8.13	44.6	54.6	25.8

[1]Days 1, 2, and 3 in the summer correspond to July 19, 21, and 23, respectively; Days 1, 2, and 3 in the fall correspond to September 12, 14, and 16, respectively.

Table 3.3. Forage nutritive values (mean ± SE)[1] on a dry matter (DM) basis for alfalfa, cool-season grass (CSG), and teff grazed by horses in July (summer) and September (fall) in St. Paul, MN during the 2016 grazing season.

Nutrient[2]	**Alfalfa**	**CSG**	**Teff**
	Summer		
DE (Mcal kg^{-1})	2.29 ± 0.01^{a}	2.24 ± 0.01^{b}	2.00 ± 0.01^{c}
Starch (%)	2.6 ± 0.5	0.6 ± 0.5	0.9 ± 0.5
WSC (%)	7.3 ± 0.6	10.6 ± 0.6	7.5 ± 0.6
ESC (%)	6.0 ± 0.5	7.6 ± 0.5	5.1 ± 0.5
NSC (%)	9.8 ± 0.6^{ab}	11.3 ± 0.6^{a}	8.4 ± 0.6^{b}
NDF (%)	46.3 ± 0.2^{c}	52.9 ± 0.2^{b}	67.0 ± 0.2^{a}
ADF (%)	34.8 ± 0.3^{a}	30.3 ± 0.3^{b}	37.1 ± 0.3^{a}
CP (%)	22.5 ± 0.5^{a}	24.1 ± 0.5^{a}	14.2 ± 0.5^{b}
	Fall		
DE (Mcal kg^{-1})	2.51 ± 0.02^{a}	2.18 ± 0.02^{b}	2.03 ± 0.02^{b}
Starch (%)	1.8 ± 0.5	0.9 ± 0.5	1.3 ± 0.5
WSC (%)	6.8 ± 0.1^{b}	9.1 ± 0.1^{a}	6.6 ± 0.1^{c}
ESC (%)	5.5 ± 0.4^{b}	7.4 ± 0.4^{a}	5.4 ± 0.4^{b}
NSC (%)	8.6 ± 0.4	9.9 ± 0.4	7.9 ± 0.4
NDF (%)	36.9 ± 0.9^{c}	55.4 ± 0.9^{b}	63.3 ± 0.9^{a}
ADF (%)	28.7 ± 0.2^{c}	31.4 ± 0.2^{b}	36.8 ± 0.2^{a}
CP (%)	27.3 ± 0.7^{a}	22.8 ± 0.7^{ab}	17.0 ± 0.7^{b}

[a-b]Within a row, means without a common letter superscript differ based on a Tukey test ($P \leq 0.05$); means without a superscript were not different ($P > 0.05$).

[1]Means were averaged within a season over three sampling days.

[2]Measured as percent DM: equine digestible energy (DE), water soluble carbohydrates (WSC), ethanol soluble carbohydrates (ESC), nonstructural carbohydrate (NSC), neutral detergent fiber (NDF), acid detergent fiber (ADF), and crude protein (CP).

Table 3.4. Glucose and insulin values (mean ± SE)[1] of horses (n = 5) grazing alfalfa, cool-season grass (CSG), and teff in July (summer) and September (fall) in St. Paul, MN during the 2016 grazing season.

Blood Characteristics	Alfalfa	CSG	Teff
	Summer		
Baseline glucose, mg dL^{-1}	88.2 ± 1.4	89.1± 1.4	88.0 ± 1.4
Average glucose, mg dL^{-1}	93.3 ± 1.3	93.7 ± 1.3	95.8 ± 1.2
Peak glucose, mg dL^{-1}	94.4 ± 2.2	100.8 ± 2.3	95.6 ± 2.2
Baseline insulin, μIU mL^{-1}	7.6 ± 1.8	7.6 ± 1.8	7.4 ± 1.8
Average insulin, μIU mL^{-1}	23.3 ± 9.3	26.2 ± 9.9	26.0 ± 9.9
Peak insulin, μIU mL^{-1}	32.3 ± 9.4	39.7 ± 9.5	32.1 ± 9.5
	Fall		
Baseline glucose, mg dL^{-1}	86.8 ± 2.0	86.7 ± 1.5	85.8 ± 1.9
Average glucose, mg dL^{-1}	94.8 ± 0.6	92.8 ± 0.5	93.6 ± 0.8
Peak glucose, mg dL^{-1}	99.5 ± 1.5	100.3 ± 1.2	97.2 ± 1.4
Baseline insulin, μIU mL^{-1}	8.6 ± 2.0	8.5 ± 1.6	8.8 ± 1.9
Average insulin, μIU mL^{-1}	41.2 ± 3.9	45.1 ± 3.3	32.7 ± 3.4
Peak insulin, μIU mL^{-1}	53.3 ± 4.1[ab]	60.5 ± 3.2[a]	39.1 ± 3.9[b]

[a-b]Within a row, means without a common letter superscript were identified as trends with a $P \leq 0.10$; means without a superscript were not different ($P > 0.10$).
[1]Means reported were averaged within a season over three sampling days.

LITERATURE CITED

Allen, E., C. Sheaffer, and K. Martinson. 2013. Forage nutritive value and preference of cool-season grasses under horse grazing. Agron. J. 105: 679-684.

Borgia, L., S.J. Valberg, K. Watts, and J. Pagan. 2009. Glycemic/insulemic response to feeding hay with different water soluble carbohydrate content in healthy and Polysaccharide Storage Myopathy-affected horses. J. Equine Vet. Sci. 29(5):355-357.

Carter, R.A., L.J. McCutcheon, L.A. George, T.L. Smith, N. Frank, and R.J. Geor. 2009. Effects of diet-induced weight gain on insulin sensitivity and plasma hormone and lipid concentrations in horses. Am. J. Vet. Res. 70:1250-1258.

Catalano, D.N., C.C. Sheaffer, A.M. Grev, M.L. Schultz, and K.L. Martinson. 2015. Forage nutritive value, yield and preference of legumes under horse grazing. J. Equine Vet. Sci. 35:435.

Chatterton, N.J., P.A. Harrison, J.H. Bennett, and K.H. Asay. 1989. Carbohydrate partitioning in 185 accessions of graminae grown under warm and cool temperatures. J. Plant Physiol. 143:169–79.

DeBoer, M.L., C.C. Sheaffer, A.M. Grev, D.N. Catalano, M.S. Wells, M.R. Hathaway, and K.L. Martinson. 2017. Yield, nutritive value, and preference of annual warm-season grasses grazed by horses. Agron. Journal. 109(5):1-13.

Forhead, A.J., and H. Dobson. 1997. Plasma glucose and cortisol responses to exogenous insulin in fasted donkeys. Res. Vet. Sci. 62:265-269.

Frank N. 2009. Insulin resistance and equine metabolic syndrome. In Robinson, N.E., and K.A. Sprayberry (eds.), Current Therapy in Equine Medicine. p. 727-740. Elsevier Publications.

Frank, N. Equine Metabolic Syndrom. 2011. Vet. Clin. North Am. Equine Pract. 27(1):73-92.

Giles, S.L., S.A. Rands, C.J. Nicol, and P.A. Harris. 2014. Obesity prevalence and associated risk factors in outdoor living domestic horses and ponies. PeerJ 2:e299.

Gordon, M.E., M.L. Jerina, C.L. King, K.E. Davison, J.K. Young, and R.H. Raub. 2007. The effects of nonstructural carbohydrate content and feeding rate on glucose and insulin response to meal feeding in equine. J. Equine Vet. Sci. 27(11):489-494.

Grev, A.M., C.C. Sheaffer, M.L. DeBoer, D.N. Catalano, and K.L. Martinson. 2017. Preference, yield, and forage nutritive value of annual grasses under horse grazing. Agron. J. 109:1-12

Hall, M.B., W.B. Hoover, J.P. Jennings, and T.K. Miller. 1999. A method for partitioning neutral detergent soluble carbohydrates. J. Sci. Food Agric. 79:2079-2086.

Henneke, D.R., G.D. Potter, J.L. Kreider, and B.F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15(4):371–372.

Jacob, S.I., R.J. Geor, P.S.D. Weber, P.A. Harris, and M.E. McCue. 2017. Effect of age and dietary carbohydrate profiles on glucose and insulin dynamics in horses. Equine Vet. J. doi:10.1111/evj.12745.

Kalu, B.A., and G.W. Fick. 1983. Morphological stage of development as a predictor of alfalfa herbage quality. Crop Sci. 23:1167-1172.

Littel, R.C., P.R. Henry, and C.B. Ammerman. 1998. Statistical anaylsis of repeated measures data using SAS procedures. J. Anim. Sci. 76: 1216-1231.

McGowan, T.W., G.P. Pinchbeck, and C.M. McGowan. 2013. Prevalence, risk factors, and clinical signs predictive for equine pituitary pars intermedia dysfunction in aged horses. Equine Vet. J. 45(1):74-79.

McGrath, D. 1992. A note on the influence of nitrogen application and time of cutting on water-soluble carbohydrate production by Italian ryegrass. Irish J. Agric. Res. 31:189-192.

McIntosh, B., D. Kronfeld, R. J. Geor, W. Staniar, A. Longland, L. Gay, D. Ward, and P. Harris. 2007. Circadian and seasonal fluctuations of glucose and insulin concentrations in grazing horses. Pages 100–101 in Proc. 20th Equine Sci. Soc. Symp., Equine Sci. Soc., Savoy, IL.

Moore, K.J., L.E. Moser, K.P. Vogel, S.S. Waller, and B.E. Johnson. 1991. Describing and quantifying growth stages of perennial forage grasses. Agron. J. 83:1073-1077.

Morgan, R.A., T.W. McGowan, and C.M. McGowan. 2014. Prevalence and risk factors for hyperinsulinaemia in ponies in Queensland Australia. Aust. Vet. J. 92:101-106.

Morin, C., G. Bélanger, G.F. Tremblay, A. Bertrand, Y. Castonguay, R. Drapeau, R. Michaud, R. Berthiaume, and G. Allard. 2011. Diurnal variations of nonstructural carbohydrates and nutritive value in alfalfa. Crop Sci. 51:1297-1306.

Morin, C., G. Bélanger, G.F. Tremblay, A. Bertrand, Y. Castonguay, R. Drapeau, R. Michaud, R. Berthiaume, and G. Allard. 2012. Short Communications: Diurnal variations of nonstructural carbohydrates and nutritive values in timothy. Can. J. Plant Sci. 92(5):883-887.

Muno, J., L. Gallatin, and R.J. Geor. 2009. Prevalence and risk factors for hyperinsulinemia in clinically normal horses in central Ohio. J. Vet. Internal Med. 23:721.

NRC. 2007. Nutrient requirements of horses. 6th ed. The National Academies Press, Washington, D.C.

Pagan, J.D. 1998. Measuring the digestible energy content of horse feeds. In: Advanced Equine Nutrition. Nottingham University Press, United Kingdom. p. 71-76.

Richards, N., and T.J. Kempton. 2016. The post feeding glycaemic and insulin response to copra meal in horses. Anim. Feed Sci. Technol. 211:100-108.

Rodiek, A.V., and C.L. Stull. 2007. Glycemic index of ten common horse feeds. J. Equine Vet. Sci. 27(5):205-212.

Rodiek, A.V., and B.E. Jones. 2012. Voluntary intake of four hay types by horses. J. Equine Vet. Sci. 32(9):579-579-583.

Schuver, A., N. Frank, K.A. Chameroy, and S.B. Elliott. 2014. Assessment of insulin and glucose dynamics by using an oral sugar test in horses. J. Equine Vet. Sci. 34(4):465-470.

Siciliano, P.D., J.C. Gill, and M.A. Bowman. 2017. Effect of sward height on pasture nonstructural carbohydrate concentrations and blood glucose/insulin profiles in grazing horses. J. Equine Vet. Sci. 57:29-34.

Staniar, W.B., T.A. Cubitt, L.A. George, P.A. Harris, and R.J. Geor. 2007. Glucose and insulin response to different dietary energy sources in thoroughbred broodmares grazing cool season pasture. Livest. Sci. 111:164-171.

Staniar, W.B., J.R. Bussard, N.M. Repard, M.H. Hall, and A.O. Burk. 2010. Voluntary intake and digestibility of teff hay fed to horses. J. Anim. Sci. 88(10):3296-3303.

Stephenson, H.M., M.J. Green, and S.L. Freeman. 2011. Short communications: prevalence of obesity in a population of horses in the UK. Vet. Rec. 168:131.

Stull, C.L., and A.V. Rodiek. 1988. Responses of blood glucose, insulin, and cortisol concentrations to common equine diets. J. Nutr. 118:206-213.

Tadros, E.M., and N. Frank. 2011. Endocrine disorders and laminitis. Equine Vet. Educ. 25(3):152-162.

Thatcher, C.D., R.S. Pleasant, R.J. Geor, and F. Elvinger. 2012. Prevalence of overconditioning in mature horses in Southwest Virginia during the summer. J. Vet. Internal Med. 26(6):1413-1418.

Vick, M.M., A.A. Adams, B.A. Murphy, D.R. Sessions, D.W. Horohov, R.F. Cook, B.J. Shelton, and B.P. Fitzgerald. 2007. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J. Anim. Sci. 85:1144-1155.

Warnken, T., K. Huber, and K. Feige. 2016 Comparison of three different methods for the quantification of equine insulin. BMC Vet. Res. 12:196-206.

Williams, C.A., D.S. Kronfeld, W.B. Staniar, and P.A. Harris. 2001. Plasma glucose and insulin responses of thoroughbred mares fed a meal high in starch and sugar or fat and fiber. J. Anim. Sci. 79:2196-2201.

Wyse, C.A., K.A. McNie, V.J. Tannahil, S. Love, and J.K. Murray. 2008. Prevalence of obesity in riding horses in Scotland. Vet. Rec. 162:590-591.

Chapter 4

Glucose and insulin response of horses grazing alfalfa, cool-season perennial grass, and teff during the extended grazing season

M.L. DeBoer[a], M.R. Hathaway[a], P.S.D. Weber[b], C.C. Sheaffer[c], K.L. Martinson[a]*

[a]Department of Animal Science, University of Minnesota, St. Paul, MN

[b]Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, MI

[c]Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN

SUMMARY

Spring and late-fall grazing can lead to metabolic problems in grazing horses (Equus caballus L.) as a result of elevated nonstructural carbohydrate (NSC) values in pasture forages. The objectives of this study were to determine the impact of different forage species on blood glucose and insulin concentrations of horses during the extended grazing season. Research was conducted in May (spring) and October (late-fall) in St. Paul, MN. Alfalfa (Medicago sativa *L.*) and mixed perennial cool-season grass (CSG) were grazed in spring and CSG and teff (*Eragrostis tef* [Zucc.] Trotter) were grazed in late-fall by six horses (24 ± 2 yr) randomly assigned to a forage in a cross-over design. Jugular catheters were inserted 1 h prior to the start of grazing and horses had access to pasture from 08:00 h to 16:00 h in the spring and 08:00 h to 12:00 h in the late-fall.

Jugular venous blood samples were collected from each horse prior to being turned out (0 h) and then at 2 hour intervals following turnout. Plasma and serum samples were collected and analyzed for glucose and insulin, respectively. Corresponding forage samples were taken by hand harvest. Seasons were analyzed separately and data were analyzed using the MIXED procedure in SAS with $P \leq 0.05$. Teff had lower NSC than CSG in the late-fall ($P \leq 0.05$) with subsequently lower average glucose, average insulin, and peak insulin in horses grazing teff compared to CSG ($P \leq 0.05$). These results suggest grazing teff could lower the glucose and insulin response of some horses.

INTRODUCTION

Forage is the predominant feed source for horses, most commonly consumed as either hay or pasture. While hay has the ability to provide forage to horses year round, fresh forage is frequently available to horses with previous studies reporting >80% of horses have some access to pasture (NAHMS, 1998; Martinson et al., 2006). Unfortunately, pasture may have a detrimental impact on grazing horses with metabolic concerns as a result of the high nonstructural carbohydrate (NSC) content in the fresh forage.

Nonstructural carbohydrates are composed of water soluble carbohydrates (WSCs), including fructans and simple sugars, as well as starch. In plants, NSCs are the main product of photosynthesis, used to provide energy for growth and metabolism in the plant. However, when photosynthesis outpaces utilization, NSCs can accumulate in forage. Numerous factors can influence NSC content including diurnal variation

(Lechtenberg et al, 1971; Holt and Hilst, 1969), forage species (Rodiek and Jones, 2012), and seasonal variation (Longland and Byrd, 2006).

Accumulation of NSCs resulting from seasonal variation is attributed to the fluctuating demands of plants for growth and development throughout the grazing season. Specifically, high NSC concentrations in forages are observed in the spring and late-fall as a result of high light intensity, resulting in increased photosynthesis, alongside low temperatures, which compromise enzymatic activity required for plant growth. Unfortunately, increased NSC concentrations are associated with a subsequent increase in the glucose and insulin response of grazing horses. While the elevated glucose and insulin response may be detrimental for grazing horses with metabolic concerns, particular forage species, including legumes (ie: alfalfa) and warm-season grasses (ie: teff,) are known to have lower NSC concentrations as a result of their self-limiting carbohydrate storage mechanism. As a result, legumes and warm-season grasses may provide an alternative forage option in comparison to cool-season grasses for horses consuming pasture during the extended grazing season. To evaluate this further, the objectives of this study were to evaluate the forage carbohydrate fractions and simultaneous glucose and insulin response of horses grazing different forage species during the spring and late-fall grazing season.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Horse Management

Six mares (24 ± 2 yr) were grazed. Horse bodyweight was 494 ± 53 kg with a BCS of 5 ± 1 (Henneke et al., 1983). One horse (Horse 6) died unexpectedly during the summer and was replaced with another horse (Horse 7) for the late-fall grazing period; the horse's death was not related to the present research.

Horses had *ad libitum* access to water throughout the study and when not grazing, horses were housed in a dry lot and fed a mixed hay containing equal parts alfalfa, CSG and teff at approximately 2.5% bodyweight (BW) split evenly between two daily feedings. Between the two grazing periods, horses grazed CSG or alfalfa pastures during the day and were housed in a dry lot overnight with *ad libitum* access to CSG hay. Horses were also fed a ration balancer (Enrich Plus Ration Balancing Horse Feed, Purina, St. Louis, MO) at 0.1% BW at 1700 h each day to ensure all vitamin and mineral requirements were met for adult horses at maintenance (NRC, 2007).

Experimental Design and Diets

Horses were randomly assigned to one of two forage types over two days in a cross-over design. Forages consisted of alfalfa, CSG (mixture of orchardgrass (*Dactylis glomerata* L.) and Kentucky bluegrass (*Poa pratensis* L.)) in the spring, and CSG and teff in the late-fall.

All pastures were mowed to 8 cm three weeks prior to the start of each grazing period to allow for an equal regrowth period. Each pasture was then divided into two equal subplots to allow each horse group (n = 3) access to fresh, un-grazed pasture during

the period. Each pasture subplot had sufficient forage available that allowed horses to graze *ad libitum* throughout the 8 hour grazing period. During the spring and late-fall, forages were grazed on May 23 and 25 and October 28 and 30, respectively. Horses grazed from 08:00 to 16:00 h in the spring and 08:00 to 12:00 in the late-fall as a result of decreased forage availability. Prior to the start of each grazing event, horses received a 24 h hay washout consisting of equal amounts of the three forage species followed by a 12 h fast. Upon completion of blood collection, horses repeated the hay washout and fasting period before switching treatments. Upon completion of grazing each day, manure was removed from the pastures and forages were mowed to 8 cm and allowed to re-grow.

Sampling and Analysis

Indwelling catheters were inserted approximately 1 h prior to the start of blood collection using a local anesthetic (2% lidocaine, Lidocaine 20 mg ml^{-1}, VetOne, MWI Animal Health. Boise, ID) blockade. Blood samples were then taken prior to turnout at 0800 (0 h) and at 2 h intervals. Serum samples were collected in 9-mL serum-separator tubes (8881302015; Covidien, Minneapolis, MN) and left at room temperature for 45 minutes following collection. Plasma samples were collected in 10-mL tubes with an EDTA-additive (8881311743; Covidien, Minneapolis, MN) and put on ice immediately after collection. Following blood collection, catheter lines were flushed with 10 mL of heparinized saline (1,000 units of heparin 200 mL^{-1} of 0.9% saline). Serum and plasma samples were separated by centrifugation at 1,200 x g at 4°C for 20 minutes, supernatants were collected, aliquoted and stored at -80°C for later analysis.

Glucose concentrations were determined in duplicate by a membrane based glucose oxidase method (YSI 2300 STAT Plus™ Glucose & Lactate Analyzer; YSI Incorporated Life Sciences, Yellow Springs, OH) using plasma samples as previously described in DeBoer et al. (2018). Insulin concentrations were determined in duplicate serum samples using the EMD Millipore Porcine Insulin Specific RIA Kit (PI-12K; EMD Millipore Porcine Insulin Specific RIA Kit; Billerica, MA, USA) previously validated for use in equine serum (Warnken et al., 2016). Intra- and inter-assay coefficients of variability (CVs) were calculated using pooled equine serum samples containing low and high concentrations of insulin. Intra- and inter-assay CVs for the low serum sample were 6.1% and 5.8%, respectively and for the high serum sample the CVs were 7.4% and 8.4%.

Duplicate, representative forage samples were taken from each pasture at 08:00, 10:00, 12:00, 14:00, and 16:00 to correspond to blood samples. Samples were clipped at 8 cm and dried for 24 h at 60º C. After drying, samples were ground through a 6-mm screen in a Wiley mill (Thomas Scientific, Swedesboro, NJ) followed by a 1-mm screen in a Cyclotec (Foss, Eden Prairie, MN). Samples were mixed thoroughly and subsamples were analyzed for CP, acid detergent fiber (ADF), neutral detergent fiber (NDF), starch, ethanol-soluble carbohydrates (ESC), WSC, NSC, and digestible energy (DE) by a commercial forage testing laboratory (Equi-Analytical, Ithaca, NY) as previously described (DeBoer et al., 2018).

Statistical Analysis

Forage qualities were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) using a repeated measures randomized complete block design. Forage, day, and forage × day were included in the model with hour used as the repeated measures. Each replicate was the experimental unit for forage. Variables analyzed included equine DE, starch, WSC, ESC, NSC, ADF, NDF, and CP.

Blood values were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) as a cross-over design. Forage, day, and sequence were included in the model. Individual horses within a forage treatment were the experimental unit for glucose and insulin concentrations. Variables analyzed included baseline, average, and peak values for both glucose and insulin in the horses. Blood samples taken at hour 0 were included in the model as a baseline covariate for peak and average glucose and insulin values. The average blood values were analyzed using a repeated measures design, with hour as the repeated measure. A square root transformation was used for peak insulin values in order to meet ANOVA assumptions; data was back transformed for presentation. Means are averaged over the sampling days within the season with the least square means of the MIXED procedure (± SE), and mean separations determined using Tukey's HSD test ($P \leq 0.05$). Based on the influencing statistics, Horse 1 was considered an outlier and was not included in the analysis. Statistical significance was set at $P \leq 0.05$.

RESULTS

Forage Nutritive Value

Differences in forage nutritive values were observed between the different species for every season (Table 1). In the spring, alfalfa had greater DE ($P \leq 0.01$), starch ($P \leq 0.01$), and ADF ($P \leq 0.01$) in comparison to CSG, but lower WSC ($P \leq 0.01$), ESC ($P \leq 0.02$), and NDF ($P \leq 0.01$). In the late fall, differences in all carbohydrate fractions were observed ($P \leq 0.05$) with lower values observed in teff compared to CSG.

Glycemic and Insulinemic Response

No differences in the glycemic or insulinemic response were observed in the spring when comparing horses grazing alfalfa or CSG ($P \geq 0.05$; Table 2). However, when evaluating horses grazing teff or CSG in the late fall, differences were observed in average glucose ($P \leq 0.01$), average insulin ($P \leq 0.01$), and peak insulin ($P \leq 0.05$). These results suggest horses grazing teff had a lower glycemic and insulinemic response when compared to horses grazing CSG.

DISCUSSION

The major finding of our study is that no differences are observed in the glycemic and insulinemic response of horses grazing alfalfa compared to CSG in the spring, however, a decreased response was observed in horses grazing teff compared to CSG in the late fall.

Forage Nutritive Values

During the spring grazing, differences between alfalfa and CSG forage nutritive values were observed. Carbohydrate fractions were different, however, WSC and ESC were higher in CSG while starch concentrations were higher in alfalfa. Higher starch concentrations observed in alfalfa were expected, as starch is the storage carbohydrate for WSGs and legumes. Similarly, elevated WSC concentrations in CSGs were expected as fructans, a component of WSCs, are their storage carbohydrate. Despite these differences, no differences were observed in forage NSC at 9.9 and 10.6% DM for alfalfa and CSG, respectively. However, increased DE observed in alfalfa needs to be taken into account for grazing horses with metabolic concerns as it could contribute to increased weight gain.

In the late-fall, more consistent differences were observed in carbohydrate fractions with lower starch, WSC, ESC, and NSC concentrations observed in teff compared to CSG. While teff, a warm-season annual grass, stores excess carbohydrates as starch in chloroplasts via a self-limiting mechanism, lower starch values in teff were likely a result of seasonal and temperature changes. Considering annual grasses only survive a single growing season, they do not have storage organs such as rhizomes, tubers, and stolons for carbohydrate reserves. As a result, when temperatures drop in the late-fall and winter, photosynthesis ceases and no more carbohydrates are produced with overall carbohydrate concentrations decreasing. While minimal research has evaluated annual warm-season grasses in the late-fall and winter, carbohydrate decreases in the

winter were observed in research evaluating bermudagrass (Dunn and Nelson, 1972). These decreased NSC concentrations could benefit horses with metabolic concerns.

Glycemic and Insulinemic Response

Glucose values observed in the current study were consistent with previous research (Stull and Rodiek, 1988; Williams et al., 2001), however, insulin values were consistently higher than values previously presented. Specifically, peak insulin values in research performed by Siciliano et al. (2017) and Staniar et al. (2007) were ≤ 43 and 31 μIU mL^{-1}, respectively. In comparison, values in the current study were higher for horses grazing alfalfa and CSG with values ≥ 58.8 μIU mL^{-1}. However, horses grazing teff had peak insulin values of 24.4 μIU mL^{-1} in the late-fall grazing which is comparable to previous research.

The elevated response in the current study could be attributed to age, as previous research has demonstrated the relationship between increased age and decreased insulin sensitivity in horses (Jacob et al., 2017). However, as an extension of the research previously reported by DeBoer et al. (2018), higher values were reported in the current study compared to the same horses evaluated during the summer and fall grazing periods. These differences were observed as average insulin values were ≤ 26.2 and 45.1 μIU mL^{-1} for the summer and fall, respectively (DeBoer et al., 2018) in comparison to the current study which observed average insulin values ≥ 51.6 μIU mL^{-1} in the spring and at 73.9 μIU mL^{-1} for CSG in the late-fall. Once again, horses grazing teff had more comparable insulin values at 16.4 μIU mL^{-1} for average insulin in the late-fall. These results suggest

an elevated insulinemic response is observed during the extended grazing season for horses grazing CSG and alfalfa.

While the NSC content of the feed has been shown to influence the glycemic and insulinemic response of horses following consumption of a meal (Staniar et al., 2007), further factors are known to play a role as well. These differences include forage management, such as sward height (Siciliano et al., 2017), other forage nutritive values, such as fiber (Richards and Kempton, 2016), and age of the horse (Jacob et al., 2017). As a result, these factors should be considered as well when determining the appropriate feed for a horse.

CONCLUSION

Research evaluating the glycemic and insulinemic response of horses grazing different forage species during the extended grazing season is limited. In the current study, higher insulin values observed in horses grazing alfalfa and CSG during the extended grazing season compared to the summer and fall in previous research (DeBoer et al., 2018), suggests horses with metabolic concerns are at greater risk when grazing during the spring and late-fall. While no differences were observed between horses grazing alfalfa and CSG in the spring, a decreased glycemic and insulinemic response was observed in horses grazing teff compared to CSG in the late-fall. As a result, while horses might demonstrate a higher insulinemic response during the extended grazing season, teff appears to offer an alternative forage source in the late-fall for horses requiring an attenuated insulin response.

Table 4.1. Forage nutritive values[1] on a dry matter (DM) basis for alfalfa, cool-season grass (CSG), and teff grazed by horses in May (Spring) and late October (Late-Fall) in St. Paul, MN during the 2016 grazing season.

Nutrient[2]	**Alfalfa**	**CSG**	**Teff**
	Spring		
DE (Mcal kg^{-1})	2.30[a]	2.17[b]	-[3]
Starch (%)	2.27[a]	0.65[b]	-
WSC (%)	7.6[b]	10.0[a]	-
ESC (%)	5.9[b]	6.9[a]	-
NSC (%)	9.9	10.6	-
NDF (%)	46.2[b]	55.9[a]	-
ADF (%)	34.9[a]	31.6[b]	-
CP (%)	21.8	22.3	-
	Late Fall		
DE (Mcal kg^{-1})	-	2.37	2.36
Starch (%)	-	1.79[a]	1.17[b]
WSC (%)	-	11.2[a]	7.2[b]
ESC (%)	-	9.7[a]	5.2[b]
NSC (%)	-	13.0[a]	8.3[b]
NDF (%)	-	47.9	46.3
ADF (%)	-	27.1[b]	31.5[a]
CP (%)	-	26.5	24.0

[a-b]Within a row, means without a common letter superscript differ based on a Tukey test ($P \leq 0.05$); means without a superscript were not different ($P > 0.05$).
[1]Means reported are averaged within a season over two or three sampling days.
[2]Measured as percent DM: digestible energy (DE), water soluble carbohydrates (WSC), ethanol soluble carbohydrates (ESC), nonstructural carbohydrate (NSC), neutral detergent fiber (NDF), acid detergent fiber (ADF), and crude protein (CP).
[3]Indicates no data available due to lack of forage mass.

Table 4.2. Glucose and insulin values (mean ± SE)[1] of non-obese (NO-OB) horses grazing alfalfa, cool-season grass (CSG), and teff in May (Spring) and late October (Late-Fall) in St. Paul, MN during the 2016 grazing season.

Blood Characteristics	**Alfalfa**	**CSG**	**Teff**
	Spring		
Baseline glucose, mg dL^{-1}	90.0 ± 2.0	92.7 ± 2.0	-[2]
Average glucose, mg dL^{-1}	104.6 ± 2.0	98.9 ± 2.0	-
Peak glucose, mg dL^{-1}	117.5 ± 2.3	109.2 ± 2.3	-
Baseline insulin, μIU mL^{-1}	11.3 ± 1.5	13.3 ± 1.5	-
Average insulin, μIU mL^{-1}	51.8 ± 11.6	47.4 ± 11.1	-
Peak insulin, μIU mL^{-1}	70.2 ± 14.8	58.8 ± 13.0	-
	Late fall		
Baseline glucose, mg dL^{-1}	-	83.7 ± 1.8	86.8 ± 1.8
Average glucose, mg dL^{-1}	-	106.0 ± 3.4[a]	94.0 ± 3.6[b]
Peak glucose, mg dL^{-1}	-	108.1 ± 3.9	98.3 ± 4.1
Baseline insulin, μIU mL^{-1}	-	11.5 ± 2.5	12.3 ± 2.5
Average insulin, μIU mL^{-1}	-	68.0 ± 13.7	19.0 ± 7.4[b]
Peak insulin, μIU mL^{-1}	-	75.0 ± 16.8[a]	24.3 ± 9.8[b]

[a-b]Within a row, means without a common letter superscript differ based on a Tukey test ($P \leq 0.05$); means without a superscript were not different ($P > 0.05$).
[1]Means reported are averaged within a season over two sampling days.
[2]Indicates no data available due to lack of forage mass.

LITERATURE CITED

DeBoer, M.L., C.C. Sheaffer, A.M. Grev, D.N. Catalano, M.S. Wells, M.R. Hathaway, and K.L. Martinson. 2017. Yield, nutritive value, and preference of annual warm-season grasses grazed by horses. Agron. Journal. 109(5):1-13.

Dunn J.H., and C.J. Nelson. 1972. Chemical changes occurring in three bermudagrass turf cultivars in relation to cold hardiness. Agron. J. 66(1):28-31.

Henneke, D.R., G.D. Potter, J.L. Kreider, and B.F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15(4):371–372.

Holt D.A., and A.R. Hilst. 1969. Daily variation in carbohydrate content of selected forage crops. Agron. J. 61(2): 239-242.

Jacob, S.I., R.J. Geor, P.S.D. Weber, P.A. Harris, and M.E. McCue. 2017. Effect of age and dietary carbohydrate profiles on glucose and insulin dynamics in horses. Equine Vet. J. doi:10.1111/evj.12745.

Lechtenberg, V.L., D.A. Holt, and H.W. Youngberg. 1971. Diurnal variation in nonstructural carbohydrates in vitro digestibility, and leaf to stem ratio of alfalfa. Agron. J. 63(5):719-724.

Longland, A.C., and B.M. Byrd. 2006. Pasture nonstructural carbohydrates and equine laminitis. J. Nutr. 136(7): 20995-21025.

Martinson, K., M. Hathaway, J.H. Wilson, B. Gilkerson, P.R. Peterson, and R.D. Vecchio. 2006. University of Minnesota Horse Owner Survey: Building an equine extension program. J. Extension. 44(6):6RIB4.

NAHMS, 1998. Equine'98, Part I: Baseline reference of 1998 equine health and management. National Animal Health Monitoring System, USDA: APHIS: VS, Fort Collins, USA, 1998.

NRC. 2007. Nutrient requirements of horses. 6th ed. The National Academies Press, Washington, D.C.

Richards, N., and T.J. Kempton. 2016. The post feeding glycaemic and insulin response to copra meal in horses. Anim. Feed Sci. Technol. 211:100-108.

Rodiek, A.V., and B.E. Jones. 2012. Voluntary intake of four hay types by horses. J. Equine Vet. Sci. 32(9):579-583.

Siciliano, P.D., J.C. Gill, and M.A. Bowman. 2017. Effect of sward height on pasture nonstructural carbohydrate concentrations and blood glucose/insulin profiles in grazing horses. J. Equine Vet. Sci. 57:29-34.

Staniar, W.B., T.A. Cubitt, L.A. George, P.A. Harris, and R.J. Geor. 2007. Glucose and insulin response to different dietary energy sources in thoroughbred broodmares grazing cool season pasture. Livest. Sci. 111:164-171.

Stull, C.L., and A.V. Rodiek. 1988. Responses of blood glucose, insulin, and cortisol concentrations to common equine diets. J. Nutr. 118:206-213.

Warnken, T., K. Huber, and K. Feige. 2016. Comparison of three different methods for quantification of equine insulin. BMC Vet. Res. 12:196-206.

Williams, C.A., D.S. Kronfeld, W.B. Staniar, and P.A. Harris. 2001. Plasma glucose and insulin responses of thoroughbred mares fed a meal high in starch and sugar or fat and fiber. J. Anim. Sci. 79:2196-2201.

BIBLIOGRAPHY

Allen, E., C. Sheaffer, and K. Martinson. 2012. Yield and persistence of cool-season grasses under horse grazing. Agron. J. 104(6):1741-1746.

Allen, E., C. Sheaffer, and K. Martinson. 2013. Forage nutritive value and preference of cool-season grasses under horse grazing. Agron. J. 105(3): 679-684.

Allen, R.E., M.H. Stromer, D.E. Goll, and R.M. Robson. 1979. Accumulation of myosin, actin, tropomyosin, and α–actinin in culture muscle cells. Dev. Biol. 69: 655-660.

Andrews, F.M., and J.A. Nadeau. 1999. Clinical syndromes of gastric ulceration in foals and mature horses. Equine Vet. J. Suppl. 29:81-85.

Askins, M.J., A.G. Palkovic, K.A. Leppo, G.C. Jones, and J.C. Gill. 2017. Effect of feeding teff hay on dry matter intake, digestible energy intake and resting insulin/glucose concentration in horses. J. Eq. Vet. Sci. 52: 45.

Badiani, A., N. Nanni, P.P. Gatta, B. Tolomelli, and M. Manfredini. 1997. Nutrient profile of horsemeat. J. Food Comp. Anal. 10: 254-269.

Balde, A.T., J.H. Vendersall, R.A. Erdman, J.B. Reeves III, and B.P. Glenn. 1993. Effect of stage of maturity of alfalfa and orchardgrass on in situ dry matter and crude protein degradability and amino acid composition. Anim. Feed Sci. Technol. 44:29-43.

Baquero-Pérez, B., S.V. Kichioudi, R.K. Nelli, and K. Chang. 2012. A simplified but robust method for the isolation of avian and mammalian muscle satellite cells. BMC Cell Biol. 13: 16.

Barzel, U.S., and L.K. Massey. 1998. Excess dietary protein can adversely affect bone. J. Nutr. 128: 1051-1053.

Borgia, L., S.J. Valberg, K. Watts, and J. Pagan. 2009. Glycemic/insulemic response to feeding hay with different water soluble carbohydrate content in healthy and Polysaccharide Storage Myopathy-affected horses. J. Equine Vet. Sci. 29(5):355-357.

Borgia L.A., S.J. Valberg, M.E. McCue, K. Watts, and J.D. Pagan. 2011. Glycemic and insulinemic responses to feeding hay with different nonstructural carbohydrate content in control and Polysaccharide Storage Myopathy-affected horses. J. Anim. Physiol. Anim. Nutr. (Berl). 95(6): 798-807.

Bott, R.C., E.A. Greene, N.L. Trottier, C.A. Williams. M.L. Westendorf, A.M. Swinker, S.L. Mastellar, and K.L. Martinson. 2016. Environmental implications of nitrogen output on horse operations: a review. J. Equine Vet. Sci. 45:98 – 106.

Bruhn, J.C., and J.C. Oliver. 1978. Effect of storage on tocopherol and carotene concentrations in alfalfa hay. J. Dairy Sci. 61: 980-982.

Buxton, D.R. 1996. Quality-related characteristics of forages as influenced by plant environment and agronomic factors. Anim. Feed Sci. Tech. 59: 37-49.

Calbet, J.A.L., and D.A. MacLean. 2002. Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans. J. Nutr. 132: 2174-2182.

Campbell-Thompson, M.L., and A.M. Merritt. 1987. Effect of ranitidine on gastric acid secretion in young male horses. Am. J. Vet. Res. 48:1511-1515.

Carter, R.A., L.J. McCutcheon, L.A. George, T.L. Smith, N. Frank, and R.J. Geor. 2009. Effects of diet-induced weight gain on insulin sensitivity and plasma hormone and lipid concentrations in horses. Am. J. Vet. Res. 70: 1250-1258.

Catalano, D.N., C.C. Sheaffer, A.M. Grev, M.L. Schultz, and K.L. Martinson. 2015. Forage nutritive value, yield and preference of legumes under horse grazing. J. Equine Vet. Sci. 35:435.

Cerling, T.E., Y. Wang, and J. Quade. 1993. Expansion of C4 ecosystems as an indicator of global ecological change in the late Miocene. Nature. 361: 344±345.

Chatterton, N.J., P.A. Harrison, J.H. Bennett, and K.H. Asay. 1989. Carbohydrate partitioning in 185 accessions of graminae grown under warm and cool temperatures. J. Plant Physiol. 143:169–79.

Connysson, M., S. Muhonen, J.E. Lindberg, B. Essen-Gustavsson, G. Nyman, K. Nostell, and A. Jansson. 2006. Effects on exercise response, fluid and acid-base balance of protein intake from forage-only diets in Standardbred horses. Equine Vet J. Suppl. 36: 648-653.

Crozier, J.A., V.G. Allen, N.E. Jack, J.P. Fontenot, and M.A. Cochran. 1997. Digestion, apparent mineral absorption, and voluntary intake by horses fed alfalfa, tall fescue, and Caucasian bluestem. J. Anim. Sci. 75:1651-1658.

Davis, T.A. and M.L. Fiorotto. 2009. Regulation of muscle growth in neonates. Curr. Opin. Clin. Nutr. Metab. Care. 12(1): 78-85.

DeBoer, M.L., C.C. Sheaffer, A.M. Grev, D.N. Catalano, M.S. Wells, M.R. Hathaway, and K.L. Martinson. 2017. Yield, nutritive value, and preference of annual warm-season grasses grazed by horses. Agron. Journal. 109(5):1-13.

DeBoer, M.L., M.R. Hathaway, K.J. Kuhle, P.S.D. Weber, A.S. Reiter, C.C. Sheaffer, M.S. Wells, and K.L. Martinson. 2018. Glucose and insulin response of horses grazing alfalfa, perennial cool-season grass, and teff across seasons. J. Equine Vet. Sci. Accepted Manuscript.

Delimaris, I. 2013. Adverse effects associated with protein intake above the recommended dietary allowance for adults. ISRN Nutr. 2013: 1-6.

DePew, C.L., D.L. Thompson, Jr., J.M. Fernandez, L.S. Sticker, and D.W. Burleigh. 1994. Changes in concentrations of hormones, metabolites, and amino acids in plasma of adult horses relative to overnight feed deprivation followed by a pellet-hay meal fed at noon. J. Anim. Sci. 72: 1530-1539.

Diaz, E.C., D.N. Herndon, C. Porter, L.S. Sidossis, O.E. Suman, and E. Borsheim. 2015. Effects of pharmacological interventions on muscle protein synthesis and breakdown in recovery from burns. Burns. 41(4): 649-657.

Dunn J.H., and C.J. Nelson. 1972. Chemical changes occurring in three bermudagrass turf cultivars in relation to cold hardiness. Agron. J. 66(1):28-31.

Elizalde, J.C., N.R. Merchen, and D.B. Faulkner. 1999. Fractionation of fiber and crude protein in fresh forages during the spring growth. J. Anim. Sci. 77:476-484.

FAO. 2001. Global estimates of gaseous emissions of NH3, NO and N2O from agricultural land, International Fertilizer Industry Association. Rome.

Ferrando, A.A., H.W. Lane, C.A. Stuart, J. Davis-Street, and R.R. Wolfe. 1996. Prolonged bed rest decreases skeletal muscle and whole body protein synthesis. Am. J. Phys. 270(4): E627-E633

Finno, C.J. E. McKenzie, S.J. Valberg, and J. Pagan. 2010. Effect of fitness on glucose, insulin, and cortisol responses to diets varying in starch and fat content in Thoroughbred horses with recurrent exertional rhabdomyolysis. Equine Vet. J. Suppl. 38: 323-328.

Fonnesbeck, P.V., M.M. Garcia de Hernandez, J.M. Kaykay, and M.Y. Saiady. 1986. Estimating yield and nutrient losses due to rainfall on field-drying alfalfa hay. Anim. Feed. Sci Technol. 16:7-15.

Forbes G.B. and J.C. Reina. 1970. Adult lean body mass declines with age: some longitudinal observations. Metabolism. 19: 653–663.

Forhead, A.J., and H. Dobson. 1997. Plasma glucose and cortisol responses to exogenous insulin in fasted donkeys. Res. Vet. Sci. 62:265-269.

Frank, N., S.B. Elliot, L.E. Brandt, and D.H. Keisler. 2006. Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. J. Am. Vet. Med. Assoc. 228: 1383-1390.

Frank N. 2009. Insulin resistance and equine metabolic syndrome. *In* Robinson, N.E., and K.A. Sprayberry (eds.), Current Therapy in Equine Medicine. p. 727-740. Elsevier Publications.

Frank, N. Equine Metabolic Syndrom. 2011. Vet. Clin. North Am. Equine Pract. 27(1):73-92.

Fry, J. and B. Huang. 2004. Applied turf grass science and physiology. Wiley, New York, NY.

Funaba, M., M. Hashimoto, C. Yamanaka, Y. Shimogori, T. Iriki, S. Ohshima, and M. Abe. 1996. Effects of a high-protein diet on mineral metabolism and struvite activity product in clinically normal cats. Am. J. Vet. Res. 57: 1726-1732.

Gates, C., and J. Huard. 2005. Management of skeletal muscle injuries in military personnel. Oper. Techn, Sports Med. 13: 247-256.

Geor, R.J., and P.A. Harris. 2009. Dietary management of obesity and insulin resistance: countering risk for laminitis. Vet. Clin. Equine. 25: 51-65.

Gibbs, P.G., G.D. Potter, G.T. Schelling, J.L. Krieder, and C.L. Boyd. 1988. Digestion of hay protein in different segments of the equine digestive tract. J. Anim. Sci. 66:400-406.

Giles, S.L., S.A. Rands, C.J. Nicol, and P.A. Harris. 2014. Obesity prevalence and associated risk factors in outdoor living domestic horses and ponies. PeerJ 2:e299.

Goodman, C.A., D.M. Mabrey, J.W. Frey, M.H. Miu, E.K. Schmidt, P. Pierre, and T.A. Hornberger. 2011. Novel insights into the regulation of skeletal muscle protein synthesis as revealed by a new nonradioactive in vivo technique. FASEB J. 25: 1028-1039.

Gordon, M.E., M.L. Jerina, R.H. Raub, K.E. Davison, and J.K. Young. 2008. The effects of feed form on consumption time and glucose and insulin response to a concentrate meal in equine. J. Eq. Vet. Sci. 28(5): 289-294.

Gordon, M.E., M.L. Jerina, C.L. King, K.E. Davison, J.K. Young, and R.H. Raub. 2007. The effects of nonstructural carbohydrate content and feeding rate on glucose and insulin

Graham-Thiers, P.M., and L.K. Bowen. 2011. Effect of protein source on nitrogen balance and plasma amino acids in exercising horses. J. Anim. Sci. 89:729-735.

Graham-Thiers, P.M., and D.S. Kronfeld. 2005. Amino acid supplementation improves muscle mass in aged and young horses. J. Anim. Sci. 83(12):2783-2788.

Graham-Thiers, P.M. D.S. Kronfield, and K.A. Kline. 1999. Dietary protein influences acid-base responses to repeated sprints. Eq. Vet. J. Suppl. 30: 463-467.

Graham-Thiers, P.M., D.S. Kronfield,, K.A. Kline, D.J. Sklan, and P.A. Harris. 2000. Protein status of exercising Arabian horses fed diets containing 14% or 7.5% crude protein fortified with lysine and threonine. J. Equine Vet. Sci. 20: 516-521.

Greene, E.A. and R.H. Raub. 1992. Procedures for harvesting satellite cells from equine skeletal muscle. J. Equine Vet. Sci. 12: 33-35.

Greger M., and G. Koneswaran. 2010. The public health impacts of concentrated animal feeding operations on local communities. Fam. Community Health. 33: 11-20.

Grev, A.M., C.C. Sheaffer, M.L. DeBoer, D.N. Catalano, and K.L. Martinson. 2017. Preference, yield, and forage nutritive value of annual grasses under horse grazing. Agron. J. 109: 1561-1572.

Gunn, H.M. 1987. Muscle, bone, and fat proportions and muscle distribution of thoroughbreds and other horses. p. 253-264. *In*: Gillespie JR, Robinson NE, editors. Equine exercise physiology 2: proceedings of the second international conference on equine exercise physiology. Davis, CA: ICEEP Publications.

Hall, M.B., W.B. Hoover, J.P. Jennings, and T.K. Miller. 1991. A method for partitioning neutral detergent soluble carbohydrates. J. Sci. Food Agric. 79:2079-2086.

Hare, P.D., and W.A. Cress. 1997. Metabolic implications of stress-induced proline accumulation in plants. J. Plant Growth Regul. 21(2):79-102.

Harkin, J.M. 1973. Lignin. P. 323 – 373. In G.E. Butler and R.W. Bailey (ed.) Chemistry and biochemistry of herbage. Academic Press. New York, NY.

Heaney, R.P. 2002. Protein and calcium: antagonists or synergists? The Am. J. of Clin. Nutr. 75: 609-610.

Henneke, D.R., G.D. Potter, J.L. Kreider, and B.F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15(4):371–372.

Heslop, L., J.E. Morgan, and T.A. Partridge. 2000. Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. J. Cell Sci. 113: 2299-2308.

Hoffman, R.M., R.C. Boston, D. Stefanovski, D.S. Kronfeld, and P.A. Harris. 2003. Obesity and diet affect glucose dynamics and insulin sensitivity in thoroughbred geldings. J. Anim. Sci. 81: 2333-2342.

Holt, D.A., and A.R. Hilst. 1969. Daily variation in carbohydrate content of selected forage crops. Agron. J. 61(2):239-242.

Huard, J., Y. Li, and F.H. Fu. 2002. Muscle injuries and repair: current trends in research. J. Bone Joint Surg. Am. 84(5): 822-830.

Hsu, J, C. Lin, and P.W. Chiou. 1998. Effects of ambient temperature and methionine supplementation of a low protein diet on the performance of laying hens. Anim. Feed Sci. Technol. 74:289-299.

Iannaccone, S.T., B. Nagy, and F.J. Samaha. 1982. Partial biochemical maturation of aneurally cultured human skeletal muscle. Neurology. 32: 846-851.

Jacob, S.I., R.J. Geor, P.S.D. Weber, P.A. Harris, and M.E. McCue. 2017. Effect of age and dietary carbohydrate profiles on glucose and insulin dynamics in horses. Equine Vet. J. doi:10.1111/evj.12745.

Johnson, R.J., and J.W. Hart. 1974. Influence of feeding and fasting on plasma free amino acids in the equine. J. Anim. Sci. 38(4): 790-794.

Johnson, C.R., B.A. Reiling, P. Mislevy, and M.B. Hall. 2001. Effects of nitrogen fertilization and harvest data on yield, digestibility, fiber, and protein fractions of tropical grasses. J. Anim. Sci. 79(9):2439-2448.

Kalu, B.A, and G.W. Fick. 1983. Morphological stage of development as a predictor of alfalfa herbage quality. Crop Sci. 23:1167-1172.

Katayama Y, Oikawa M, Yoshihara T, Kuwano A, Hobo S. 1995. Clinico-Pathological Effects of Atmospheric Ammonia Exposure on Horses. J. Equine Vet. Sci. 6: 99-104.

Kerr, B.J., F.K. McKeith, and R.A. Easter. 1995. Effect on performance and carcass characteristics of nursery to finisher pigs fed reduced crude protein, amino acid-supplemented diets. J. Anim. Sci. 73:433-440.

Kim, K.I., S. Lee, and R. Choue. 2011. Metabolic responses to high protein diet in Korean elite bodybuilders with high-intensity resistance exercise. J. of the Int. Soc. Of Sports Nutr. 8.

Kronfeld, D.S., K.H. Treiber, T.M. Hess, and R.C. Boston. 2005. Insulin resistance in the horse: definition, detection and dietetics. J. Anim. Sci. 83: E22-E31.

LaCasha, P.A., H.A. Brady, V.G. Allen, C.R. Richardson, and K.R. Pond. 1999. Voluntary intake, digestibility, and subsequent selection of Matua bromegrass, coastal bermudagrass, and alfalfa hays by yearling horses. J. Anim. Sci. 77:2766-2773.

Lechtenberg, V.L., D.A. Holt, and H.W. Youngberg. 1971. Diurnal variation in nonstructural carbohydrates in vitro digestibility, and leaf to stem ratio of alfalfa. Agron. J. 63(5):719-724.

Littel, R.C., P.R. Henry, and C.B. Ammerman. 1998. Statistical anaylsis of repeated measures data using SAS procedures. J. Anim. Sci. 76: 1216-1231.

Longland, A.C., and B.M. Byrd. 2006. Pasture nonstructural carbohydrates and equine laminitis. J. Nutr. 136(7): 20995-21025.

Martinson, K.L., M.S. Wells, and C.C. Sheaffer. 2016. Horse preference, forage yield, and species persistence of 12 perennial cool-season perennial grass mixtures under horse grazing. J. Equine Vet. Sci. 36: 19-25.

Martinson, K., M. Hathaway, J.H. Wilson, B. Gilkerson, P.R. Peterson, and R.D. Vecchio. 2006. University of Minnesota Horse Owner Survey: Building an Equine Extension Program. Journal of Extension. 44(6): 6RIB4.

Mastellar, S.L, A. Moffet, P. Harris, and K.L. Urschel. 2016. Effects of threonine supplementation on whole-body protein synthesis and plasma metabolites in growing and mature horses. The Vet. J. 126: 93-100.

Mauro, A. 1961, Satellite cell of skeletal muscle fibers. J. Biophys. Biochem. Cytol. 9: 492-495.

McCormick, J.S., R.M. Sulc, D.J. Barker, and J.E. Beuerlein. 2006. Yield and nutritive value of autumn-seeded winter-hardy and winter-sensitive annual forages. Crop Sci. 46(5): 1981.

McCowan, S., M. Brummer, S. Hayes, G. Olson, S.R. Smith, and L. Lawrence. 2012. Acceptability of teff hay by horses. J. Equine Vet. Sci. 32:327-331.

McCrory, D.F., and P.J. Hobbs. 2001. Additives to reduce ammonia and odor emissions from livestock wastes. A review. J. Environ. Qual. 30: 345-355.

McCue, M.E., R.J. Geor, and N. Schultz. 2015. Metabolic syndrome: a complex disease influenced by genetics and the environment. J. Equine Vet. Sci. 35: 367-375.

McGowan, T.W., G.P. Pinchbeck, and C.M. McGowan. 2013. Prevalence, risk factors, and clinical signs predictive for equine pituitary pars intermedia dysfunction in aged horses. Equine Vet. J. 45(1):74-79.

McGrath, D. 1992. A note on the influence of nitrogen application and time of cutting on water-soluble carbohydrate production by Italian ryegrass. Irish J. Agric. Res. 31:189-192.

McIntosh, B., D. Kronfeld, R. J. Geor, W. Staniar, A. Longland, L. Gay, D. Ward, and P. Harris. 2007. Circadian and seasonal fluctuations of glucose and insulin concentrations in grazing horses. Pages 100–101 in Proc. 20th Equine Sci. Soc. Symp., Equine Sci. Soc., Savoy, IL.

Miller, P.A., and L.M. Lawrence. 1988. The effect of dietary protein level on exercising horses. J. Anim. Sci. 66: 2185-2192.

Minty, A.J., S. Alonso, M. Caravatti, and M.E. Buckingham. 1982. A fetal skeletal muscle actin mRNA in the mouse and its identity with cardiac actin mRNA. Cell 30: 185-192.

Moore, J.E. and G.O. Mott. 1973. Structural inhibitors of quality tropical grasses. *In* Matches, A.G. (ed.), Anti-quality components of forages. p. 53-98. Madison: Crop Science Society of America.

Moore, K.J., L.E. Moser, K.P. Vogel, S.S. Waller, and B.E. Johnson. 1991. Describing and quantifying growth stages of perennial forage grasses. Agron. J. 83:1073-1077.

Moore, K.J., and H.G. Jung. 2001. Lignin and Fiber Digestion. J. Range Managem. 54(4):420-430.

Moore, K.J., T.A. White, R.L Hintz, P.K. Patrick, and E.C. Brummer. 2004. Sequential grazing of cool- and warm-season pastures. Agron. J. 96:1103-1111.

Morgan, R.A., T.W. McGowan, and C.M. McGowan. 2014. Prevalence and risk factors for hyperinsulinaemia in ponies in Queensland Australia. Aust. Vet. J. 92:101-106.

Morin, C., G. Bélanger, G.F. Tremblay, A. Bertrand, Y. Castonguay, R. Drapeau, R. Michaud, R. Berthiaume, and G. Allard. 2011. Diurnal variations of nonstructural carbohydrates and nutritive value in alfalfa. Crop Sci. 51:1297-1306.

Morin, C., G. Bélanger, G.F. Tremblay, A. Bertrand, Y. Castonguay, R. Drapeau, R. Michaud, R. Berthiaume, and G. Allard. 2012. Short Communications: Diurnal variations of nonstructural carbohydrates and nutritive values in timothy. Can. J. Plant Sci. 92(5):883-887.

Moss, F.P. and C.P. Leblond. 1971. Satellite cells as a source of nuclei in muscles of growing rats. Anat. Rec. 170: 421-436.

Mowat, D.N., R.S. Fulkerson, W.E. Tossell, and J.E. Winch. 1965. The in vitro digestibility and protein content of leaf and stem portions of forages. Can. J. Plant Sci. 45:321-331.

Muno, J., L. Gallatin, and R.J. Geor. 2009. Prevalence and risk factors for hyperinsulinemia in clinically normal horses in central Ohio. J. Vet. Internal Med. 23:721.

Murray, M.J. 1992. Gastric ulceration in horses: 91 cases (1987 – 1990). J. Am. Vet. Med. Ass. 201:117-120.

Murray, M.J., and G.F. Schusser. 1993. Measurement of 24-h gastric pH using an indwelling pH electrode in hroses unfed, fed, and treated with ranitidine. 25(5): 417-421.

Mus, F., M.B. Crook, K. Garcia, A.Garcia Costas, B.A. Geddes, E.D. Kouri, P. Paramasivan, M. Ryu, G.E.D. Oldroyd, P.S. Poole, M.K. Udvardi, C.A. Voigt, J. Ané, and J.W. Peters. 2016. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Appl. Environ. Microbiol. 82(13): 3698-3710.

Myburgh, K.H., J. Hutchins, A.B. Fataar, S.F. Hough, and T.D. Noakes. 1990. Low bone density is an etiologic factor for stress fractures in athletes. Ann. Intern. Med. 113: 754-759.

NAHMS, 1998. Equine'98, Part I: Baseline reference of 1998 equine health and management. National Animal Health Monitoring System, USDA: APHIS: VS, Fort Collins, USA, 1998.

Nakano, K. and H. Hara. 1979. Measurement of the protein-synthetic activity in vivo of various tissues in rats by using [^{3}H]puromycin. Biochem J. 184: 663-668.

Nathans, D. 1964. Puromycin inhibition of protein synthesis: incorporation of puromycin into peptide chains. Proc. Natl. Acad. Sci. U.S.A. 51: 585-592.

Nicholson, S.S. 2007. Nitrate and nitrite accumulating plants. In: R.C. Gupta, editor, Veterinary toxicology, basic clinical principles. Elsevier. Amsterdam, the Netherlands. p. 876 – 879.

Nielsen, B.D., C.I. O'Connor-Robison, H.S. Spooner, and J. Shelton. 2010. Glycemic and insulinemic responses are affected by age of horse and method of feed processing. J. Eq. Vet. Sci/ 30(5): 249-258

NRC. 1989. Nutrient Requirements of Horses. 5th ed. The National Academy Press, Washington D.C.

NRC. 2007. Nutrient Requirements of Horses. 6th ed. The National Academy Press, Washington, D.C.

Orton, R.K., I.D. Hume, and R.A. Leng. 1985. Effects of level of dietary protein and exercise on growth rates of horses. Equine Veterinary Journal. 17: 381-385.

Pagan, J.D. Measuring the digestible energy content of horse feeds. In Advanced Equine Nutrition. Inited Kingdom: Nottingham University Press; 1998. p. 71-76.

Palmonari, A., M. Fustini, G. Canestrari, E. Grili, and A. Formigoni. 2014. Influence of maturity on alfalfa hay nutritional fractions and indigestible fiber content. J. Dairy Sci. 97:7729-7734.

Patterson, P.H., C.N. Coon, and I.M. Hughes. 1985. Protein requirements of mature working horses. J. Anim. Sci. 61: 187-196.

Perriard, J.-C., U.B. Rosenberg, T. Walliman, H.M. Eppenberger, and M. Caravatti. 1982. The switching of creatine kinase gene expression during myogenesis. *In* Pearson, M.L. and H.F. Epstein (eds.), Muscle Development: Molecular and Cellular Control. p. 237-245. Cold Spring Harbor Laboratory, New York.

Ralston, S.L. 1996. Hyperglycemica/hyperinsulinemia after feeding a meal of grain to young horses with osteochondritis dissecans (OCD) lesions. Pferdeheilkunde. 12: 320-322.

Reid, R.L., G.A. Jung, and W.V. Thayne. 1988. Relationships between nutritive quality and fiber components of cool season and warm season forages: a retrospective study. J. Anim. Sci. 66: 1275-1291.

Rennie, M.J. and K.D. Tipton. 2000. Protein and amino acid metabolism during and after exercise and the effects of nutrition. Annu. Rev. Nutr. 20: 457-483.

Richards, N., and T.J. Kempton. 2016. The post feeding glycaemic and insulin response to copra meal in horses. Anim. Feed Sci. Technol. 211:100-108.

Riesterer, J.L., M.D. Casler, D.J. Undersander, and D.K. Combs. 2000. Seasonal yield distribution of cool-season grasses following winter defoliation. Agron. J. 92: 974-980.

Rodiek, A.V., and C.L. Stull. 2007. Glycemic index of ten common horse feeds. J. Equine Vet. Sci. 27(5):205-212.

Rodiek, A.V., and B.E. Jones. 2012. Voluntary intake of four hay types by horses. J. Equine Vet. Sci. 32(9):579-583.

Roughead, Z.K. 2003. Is the interaction between dietary protein and calcium destructive or constructive for bone?: Summary. J. Nutr. 133: 866S-869S.

Rotz, C.A., and R.E. Muck. 1994. Changes in forage quality during harvest and storage. G.C. Fahey, M. Collins, D.R. Mertens, L.E. Moser (Eds.), Forage Quality, Evaluation, and Utilization, American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wisc., USA (1994), pp. 828-868.

Russell, M.A., A.V. Rodiek, and L.M. Lawrence. 1986. Effect of meal schedules and fasting on selected plasma free amino acids in horses. J. Anim. Sci. 63: 1428-1431.

Sage, R., and D. Kubien. 2003. Quo vadis C4? An ecophysiological perspective on global change and the future of C4 plants. Photosynth. Res. 77: 209–225.

Salon, C., N. Munier-Jolain, G. Duc, A. Voisin, D. Grandgirard, A. Larmure, R.J.N. Emery, and B. Ney. 2001. Grain legume seed fillin in relation to nitrogen acquisition: A review and prospects with particular reference to pea. Agronomie. 21:539-552.

Scharner, J., and P.S. Zammit. 2011. The muscle satellite cell at 50: the formative years. Skeletal muscle 1:28.

Schiaffino, S., S. Pierobon Bormioli, and M. Aloisi. 1976. The fate of newly formed satellite cells during compensatory muscle hypertrophy. Virchows Arch. B. Cell Pathol. 21: 113-118.

Schiffman S., C. Studwell, L. Landerman, K. Berman, and J. Sundy. 2005. Symptomatic effects of exposure to diluted air sampled from a swine confinement atmosphere on healthy human subjects. Environ. Health Persp. 113: 567-576.

Schmidt, E.K., G. Clavarino, M. Ceppi, and P. Pierre. 2009. SUnSET, a nonradioactive method to monitor protein synthesis. Nature Methods. 6(4): 275-277.

Schultz, E., M.C. Gibson, T. Champion. 1978. Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. J. Exp. Zool. 206: 451-456.

Schuver, A., N. Frank, K.A. Chameroy, and S.B. Elliott. 2014. Assessment of insulin and glucose dynamics by using an oral sugar test in horses. J. Equine Vet. Sci. 34(4):465-470.

Sharpley, A., S. Chapra, R. Wedepohl, J. Sim. T. Daniel, K. Reddy. 1994. Managing agricultural phosphorus for protection of surface waters: Issues and options. J. Environ. Qual. 23: 437-451.

Sheffield, R. 2012. Atmospheric Ammonia: Understand Its Effects. Louisiana State University Extension.

Shepherd, M.L., R.S. Pleasant, M.V. Crisman, S.R. Were, S.C. Milton, and W.S. Swecker Jr. 2012. Effects of high and moderate non-structural carbohydrate hay on insulin, glucose, triglyceride, and leptin concentrations in overweight Arabian geldings. J. Anim. Phys. Anim. Nutr. 96: 428-435.

Siciliano, P.D., J.C. Gill, and M.A. Bowman. 2017. Effect of sward height on pasture nonstructural carbohydrate concentrations and blood glucose/insulin profiles in grazing horses. J. Equine Vet. Sci. 57:29-34.

Sleugh, B., K.J. Moore, J.R. George, and E.C. Brummer. 2000. Binary legume-grass mixtures improve forage yield, quality, and seasonal distribution. Agron. J. 92:24-29.

Soeta, C. K. Yamanouchi, T. Hasegawa, N. Ishida, H. Mukoyama, H. Tojo, and C. Tachi. 1998. Isolation of satellite cells from equine skeletal muscle. J. Equine Vet. Sci. 9(3): 97-100.

Staniar, W.B., T.A. Cubitt, L.A. George, P.A. Harris, and R.J. Geor. 2007. Glucose and insulin response to different dietary energy sources in thoroughbred broodmares grazing cool season pasture. Liv. Sci. 111: 164-171.

Staniar, W.B., J.R. Bussard, N.M. Repard, M.H. Hall, and A.O. Burk. 2010. Voluntary intake and digestibility of teff hay fed to horses. J. Anim. Sci. 88:3296-3303.

Steinfeld, H., P. Gerber, T. Wassenaar, V. Castel, M. Rosales, and C. de Haan. 2006. Livestock's long shadow: Environmental issues and options. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Stephenson, H.M., M.J. Green, and S.L. Freeman. 2011. Short communications: prevalence of obesity in a population of horses in the UK. Vet. Rec. 168:131.

Sticker, L.S., D.L. Thompson Jr., L.D. Bunting, J.M. Fernandez, and C.L. DePew. 1995. Dietary protein and(or) energy restriction in mares: plasma glucose, insulin, nonesterified fatty acid, and urea nitrogen responses to feeding, glucose, and epinephrine. J. Anim. Sci. 73: 136-144.

Straková, E., P. Suchý, I. Král, D. Zapltal, and I. Herzig. 2013. The content of amino acids in pasture vegetation and their apparent digestibility in 2-year-old horses. J. Equine Vet. Sci. 33:984-988.

Stull, C.L. and A.V. Rodiek. 1988. Responses of blood glucose, insulin, and cortisol concentrations to common equine diets. J. Nutr. 118: 206-213.

Svanberg, E., L.S. Jefferson. K. Lundholm, and S.R. Kimball. 1997. Postprandial stimulation of muscle protein synthesis is independent of changes in insulin Am. J. Physiol. 272: E841-E847.

Tadros, E.M., and N. Frank. 2011. Endocrine disorders and laminitis. Equine Vet. Educ. 25(3):152-162.

Tanner, S.L., A.L. Wagner, R.N. Digianantonio. P.A. Harris, J.T. Sylvester, and K.L. Urschel. 2014. Dietary crude protein intake influences rates of whole-body protein synthesis in weanling horses. Vet. J. 202: 236-243.

Thatcher, C.D., R.S. Pleasant, R.J. Geor, and F. Elvinger. 2012. Prevalence of overconditioning in mature horses in Southwest Virginia during the summer. J. Vet. Internal Med. 26(6):1413-1418.

Trieber, K.H., D.S. Kronfeld, and R.J. Geor. 2006. Insulin resistance in equids: possible role in laminitis. J. Nutr. 136: 2094S-2098S.

Trottier, N.L., B.D. Nielsen, K.J. Lang, P.K. Ku, and H.C. Schott. 2002. Equine endurance exercise alters serum branched-chain amino acid and alanine concentrations. Equine Vet. J. 34: 168-172.

Umpleby, A.M. and D.L. Russell-Jones. 1996. The hormonal control of protein metabolism. Bailliere Clin. Endoc. 10(4): 551-570.

Van der Eerden, L.J.M., P.H.B de Visser, and C.J. Van Dijk. 1998. Rise of damage to crops in the direct neighborhood of ammonia sources. Environ. Pollut. 102(51): 49-53.

Van Eps, A.W., and C.C. Pollitt. 2006. Equine laminitis induced with oligofructose. Equine Vet. J. 38(3):203-208.

Vick, M.M., A.A. Adams, B.A. Murphy, D.R. Sessions, D.W. Horohov, R.F. Cook, B.J. Shelton, and B.P. Fitzgerald. 2007. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J. Anim. Sci. 85: 1144-1155.

Warnken, T., K. Huber, and K. Feige. 2016 Comparison of three different methods for the quantification of equine insulin. BMC Vet. Res. 12:196-206.

Weir, W.C., L.G. Jones, and J.H. Meyer. 1960. Effect of cutting interval and stage of maturity on the digestibility and yield of alfalfa. J. Anim. Sci. 19(1): 5-19.

Weyenberg, S.V., J. Sales, and G.P.J. Janssens. 2006. Passage rate of digesta through the equine gastrointestinal tract: a review. Livestock Sci. 99(1): 3 – 12.

Whalen, R.G. 1980. Contractile protein isozymes in muscle development: The embryonic phenotype. *In* Pette, D. (ed.), Plasticity of Muscle. p. 177-191. Walter de Gruyter, Berlin.

Wickens, C.L., J. Moore, J. Shelle, C. Skelly, and N.L. Trottier. 2003. Effect of exercise on dietary protein requirement of the Arabian horse. In: Proc. Eq. Nutr, Physiol. Society, East Lansing, MI. 18:129-131.

Wickens, C.L., J. Moore, C. Wolf, C. Skelley, and N. Trottier. 2005. 3-methylhistidine as a response criterium to estimate dietary protein requirement of the exercising horse. In: Proc. Eq. Nutr. Physiol. Society. 19:205.

Willard, J.G., J.C. Willard, S.A. Wolfram, and J.P. Baker. 1977. Effect of diet on cecal pH and feeding behavior of horses. J. Anim. Sci. 45:87-93.

Williams, C.A., D.S. Kronfeld, W.B. Staniar, and P.A. Harris. 2001. Plasma glucose and insulin responses of thoroughbred mares fed a meal high in starch and sugar or fat and fiber. J. Anim. Sci. 79:2196-2201.

Williams C.A., L.B. Kenny, and A.O. Burk. 2017. Effects of grazing system and season on glucose and insulin dynamics of the grazing horse. J. Eq. Vet. Sci. 52: 87.

Wilson, J.R. 1994. Cell wall characteristics in relation to forage digestion by ruminants. J. Agr. Sci. 122:173-182.

Woodward, A.D., B.D. Nielsen, J. Liesman, T. Lavin, and N.L. Trottier. 2011. Protein quality and utilization of timothy, oat-supplemented timothy, and alfalfa at differing harvest maturities in exercised Arabian horses. J. Anim. Sci. 89:4081-4092.

Wyse, C.A., K.A. McNie, V.J. Tannahil, S. Love, and J.K. Murray. 2008. Prevalence of obesity in riding horses in Scotland. Vet. Rec. 162:590-591.

Yoshizawa, F., S.R. Kimball, T.C. Vary, and L.S. Jefferson. 1998. Effect of dietary protein on translation initiation in rat skeletal muscle and liver. Am. J. Physiol. 275: E814-E820.

Zhu, G., H. Xiao, Q. Guo, Z. Zhang, J. Zhao, and D. Yang. 2018. Effects of cadmium stress on growth and amino acid metabolism in two compositae plants. Ecotoxicol. Environ. Saf. 158:300-308.